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BOTULINUM TOXIN (TYPE A); INCLUDING A STUDY OF SHAKING WITH CHLOROFORM AS A STEP IN THE ISOLATION PROCEDURE[1]

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The type A toxin of *Clostridium botulinum* has been isolated and crystallized. It has proved to be a heat-coagulable protein having the solubility properties of a globulin. A step-by-step description of the purification procedure has been reported elsewhere (Lamanna *et al.*, 1946). It is the object of the present paper to review some of the data which support the validity of the method and to discuss the properties of the toxin at various stages of purity. Special attention is devoted to shaking with chloroform as a method for the isolation of a protein. Abrams *et al.* (1946) have also reported a method for the purification of this toxin.

In 1928 Snipe and Sommer reported the concentration of the toxin from the medium by acid precipitation. Subsequently Sommer (1937) found that the toxin could be extracted from the precipitate of acid-treated whole culture by resuspension of the acid precipitate in 1 per cent (0.075 M) sodium acetate solution at pH 6.5 and centrifugation at this pH value to remove the bacterial cells and other insoluble matter. Carrying the purification beyond these first two steps has made possible the final isolation of the toxin. The new steps include extraction of the toxin from the acid precipitate of whole culture by resuspension in a mixture of molar sodium chloride and 0.075 M sodium acetate at pH 6.5, shaking with chloroform, reprecipitation with acid, and fractionation by salting out with ammonium sulfate in the presence of molar sodium chloride at controlled pH values.

The Hall strain of type A *Clostridium botulinum* was used because of its consistent high-titer production of toxin. The medium, prepared in 16-liter lots in 5-gallon pyrex carboys, was composed of 0.3 per cent casein (tech. grade), 0.5 per cent glucose, and 1 per cent alkaline-treated corn steep liquor (47 to 52 per cent solids) adjusted to pH 7.2 before sterilization. Sterilization was accomplished by holding the carboys for 1 hour in the autoclave after the steam pressure had risen to 15 pounds. The quantity of casein used was the minimum amount that would support a high yield of toxin (800,000 to 1,500,000 MLD[4] per ml). Incubation at 34 C was conducted for 80 hours, after which time no further increase in toxin titer could be expected. A more complete discussion of the factors concerned in the production of toxin in this type of medium will be found in the paper by Lewis and Hill (1946). The successful use of this economical and highly complex nonsynthetic medium for the isolation of a bacterial toxin shows that synthetic media are not absolutely necessary for this purpose. Of far greater importance is the use of a bacterial strain that will produce an abundance of toxin.

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[1] Studies conducted at Camp Detrick, Frederick, Maryland, from June, 1944, to December, 1945.

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[4] MLD refers to intraperitoneal injection into 20-gram white mice (±2 g) of the least amount of material killing all mice tested within 4 days.

Acid precipitation of the 80-hour culture was effected by bringing the pH to 3.5 with 2 N hydrochloric acid. It is rather remarkable that concentrated hydrochloric acid (sp gr 1.09) could be used at this stage without any apparently harmful effect. Usually, 20 five-gallon carboys of culture were prepared at one time, and the acid precipitates from each were pooled for further processing.

For recovery of the toxin from the acid mud the use of an extractive solution of a mixture of molar sodium chloride and 0.075 M sodium acetate, instead of acetate alone as recommended by Sommer (1937), was found to be superior for two reasons. In the first place the efficiency of extraction can be increased by raising the pH. The toxin, however, is extremely sensitive to pH values above 6.5 to 7.0 at room temperature, the temperature at which the present work was done. It was found that the sensitivity to alkali was decreased by the addition of a variety of inorganic salts including sodium chloride, sodium sulfate, ammonium sulfate, magnesium sulfate, and monobasic ammonium phosphate. At the same time the increased salt concentration brought more toxin into solution relative to the total amount of redissolved nitrogenous compounds. Table 1 illustrates these relationships using 0.075 M sodium acetate and 20 per cent saturation with sodium sulfate as contrasted examples. Secondly, the presence of molar sodium chloride reduces to about half the degree of saturation with ammonium sulfate required for salting out the toxin at all pH values studied (4.0 to 7.0). The lesser amount of ammonium sulfate required for salting out results in less extraneous protein and nucleic acid accompanying the toxin.

SHAKING WITH CHLOROFORM IN THE PURIFICATION OF TOXIN

It has been a common observation (Sevag, 1934; Sevag and Smolens, 1941) that shaking protein solutions with chloroform will cause the appearance of a gel that that can be centrifuged off. In this fashion it is possible to deproteinize solutions. The influence of chloroform shaking on the dissolved toxin in the acetate extract of the acid precipitate of whole culture was studied. The purpose of the work was to attempt to separate the toxin from other proteins. This was at a stage in the investigation when the evidence was not complete that the toxin was a protein. It soon developed that a valuable function could be served by chloroform shaking, namely, the pH of acid precipitation of the toxin in 0.075 M sodium acetate could be changed to 5. This permitted separation of the toxin from inert proteins and nucleic acid soluble at this pH.

When chloroform was shaken with the toxin solution and centrifuged, three layers resulted: a top toxic solution, an intermediate layer of toxic protein gel, and a bottom layer of excess chloroform. In the refrigerator a toxic aqueous solution was liberated from the gel. The syneresis permitted maximum recovery of toxin from the chloroform-protein gel.

A study was undertaken of some of the variables influencing toxin recovery after chloroform shaking. Equivalent samples of acetate (0.075 M) extracts of acid mud were adjusted to different pH values and shaken by hand with one-fourth their volumes of cp chloroform for 5 minutes in corked flasks. The mixtures were centrifuged and separated into three layers: a top aqueous layer, a middle layer of gelatinous material, and a bottom layer of excess chloroform. Toxin titers and pH determinations indicated that, depending on the pH of the extract before shaking, the toxin was detoxified, remained active and in solution, or remained active and precipitated as a part of the gel. Thus acetate extracts adjusted initially to pH values of 6.8, 6.5, 6.15, 5.9, 5.7, and 5.45 rose to pH 7.4, 7.08, 6.6, 6.1, 5.8, and 5.52 after shaking. With a starting pH of 5.7 or lower, active toxin tended to come out in the gel. Between pH 5.7 and 6.5 the bulk of the toxin remained active and in solution. When the pH after shaking was 6.5 or greater, the toxin did not come down with the gel, but tended to be detoxified and to remain in aqueous solution.

TABLE 1

Influence of pH and salt concentration on the extraction of toxin from an acid precipitate of whole culture, and its subsequent stability

pH		DILUTION IN MILLIONS OF SOLUTION KILLING 20-GRAM MICE									MG NITROGEN PER ML SOLUTION
Extraction	On 3 days' storage	After extraction			After 3 days at room temperature			After 10 days at room temperature			
		5	10	20	5	10	20	5	10	20	
0.075 M N_a-acetate											
6.5	6.6	4/4*	4/4	0/4	4/4	4/4	1/4	0/4	0/4	0/4	0.706
6.5	6.55	3/4	3/4	0/4	3/4	3/4	3/4	0/4	0/4	0/4	0.760
8.5	7.45	4/4	3/4	0/4	0/4	0/4	0/4				0.854
8.5	7.4	2/4	3/4	2/4	0/4	1/4	0/4				0.875
8.5	7.3	4/4	4/4	1/4	0/4	0/4	0/4				0.909
9.5	7.7	4/4	4/4	0/4	0/4	0/4	0/4				0.919
9.5	7.65	3/4	4/4	0/4	0/4	0/4	0/4				0.944
9.5	7.6	4/4	4/4	0/4	0/4	0/4	0/4				0.955
20% sat. Na_2SO_4											
6.5	6.7	4/4	2/4	0/4	4/4	4/4	1/4	3/4	3/4	0/4	0.571
6.5	6.6	3/4	3/4	0/4	4/4	4/4	4/4	2/4	3/4	0/4	0.577
8.5	7.4	4/4	4/4	4/4	4/4	3/4	0/4				0.798
8.5	7.5	3/4	4/4	3/4	4/4	4/4	3/4				0.668
8.5	7.5	4/4	4/4	3/4	3/4	4/4	3/4				0.654
9.5	7.9	4/4	4/4	3/4	4/4	4/4	4/4				0.681
9.5	7.9	3/4	3/4	4/4	4/4	4/4	4/4				0.728
9.5	7.8	4/4	4/4	4/4	4/4	4/4	3/4				0.757

$$* \text{ Ratio} = \frac{\text{no. of mice killed}}{\text{no. of mice injected}}.$$

The observed change in pH that accompanies the chloroform shaking is influenced by the buffer capacity of the medium. Thus, by shaking under a CO_2 atmosphere the direction of the change can be reversed. The degree to which the pH change occurs when CO_2 is employed was found to be dependent upon the concentration of the sodium acetate present in the solution. Table 2 illustrates the findings with CO_2.

TABLE 2

Influence of CO_2 on pH change accompanying chloroform shaking

ATMOSPHERE	MOLARITY OF SODIUM ACETATE	pH OF ACETATE EXTRACT			pH OF GEL*
		Before shaking	After shaking		
			Before removing gel	After removing gel	
CO_2	0.75	6.5	6.39		
		6.1	5.55	5.7	5.7
	0.075	6.45	6.07		
		6.7	5.9	6.05	
Air	0.075	6.1	6.4	6.35	6.18
N_2	0.075	6.1	6.37	6.35	6.15

* Resuspended to original volume in distilled water.

TABLE 3

Influence of NaCl on the chloroform-shaking step in toxin purification

CONCENTRATION OF NaCl	GASEOUS ENVIRONMENT DURING SHAKING	pH BEFORE SHAKING	pH AFTER SHAKING	TOXIN TITER OF EXTRACT AS PERCENTAGE RECOVERED
		6.8	7.0	1
0	Air	6.7	6.8	0.4
		6.5	6.65	100
		6.0	6.1	50
Molar	CO_2	5.8	5.0	100
		6.58	6.6	100
		6.5	6.5	90
	Air	6.5	6.5	100
		5.55	5.6	100
		5.0	5.0	100
2 Molar	CO_2	6.5	5.9	4
	Air	6.5	6.5	90

Salt concentration also plays a role. Addition of sodium chloride to molar concentration extended the pH range at which the toxin solution might be shaken with chloroform with recovery of active toxin in the aqueous phase, and decreased the extent of the pH change accompanying shaking (table 3). Too few runs were tested at 2 M sodium chloride to draw any conclusions; but if the tests are indicative, this concentration has a narrower pH range for recovery of active toxin in the aqueous phase than has the molar concentration.

When detoxification occurs by reason of shaking with chloroform at a high pH value, although a loss in pharmacological activity is noted, an equivalent loss in ability to combine with commercially prepared horse antitoxin is not evident. Tests on batches of acetate extract similarly treated, except for differences in shaking conditions, that resulted in losses as great as 87 to 97 per cent of the toxicity did not show differences in flocculation values. These toxin solutions did not precipitate normal horse serum, which was used as a control. If the detoxified toxin still capable of combining with antitoxin has immunizing properties, toxoid formation has occurred. This problem is being studied in further investigations.

An experiment was performed to determine the effect of repeated shakings with chloroform. Forty ml of solution of toxin, which had been purified by two shakings with chloroform and had an LD50 of 20 million per ml, were brought to pH 5.0 and shaken for 15 minutes with 10 ml of freshly distilled chloroform under an atmosphere of carbon dioxide. A considerable amount of gel was removed by centrifugation. This process was repeated 8 times with fresh chloroform. The gel resulting from the third shaking was the most voluminous, after which the gels became continually smaller in amount. On titrating for toxin after the sixth and ninth shakings with chloroform, the water layer was found to contain less than 200,000 LD50 per ml. Hence, there is almost a complete removal of toxin from the solution upon repeated chloroform shakings. This is essentially the procedure that would be used for complete deproteinization of solutions.

The gel formed by shaking with chloroform. When the acetate extract is shaken with chloroform, some protein comes down in the gel. The pH at which shaking takes place evidently affects the kind of protein being brought down. This point is illustrated in the following experiment: A batch of 0.075 M sodium acetate extract was divided into eight 50-ml samples. Each sample was adjusted to a different pH in the range between 7 and 3.25. Then each batch was shaken under air with one-fourth its volume of cp chloroform for 5 minutes. After centrifugation to remove excess chloroform and the gel, the resultant aqueous supernatant was brought down with acid to the pH at which turbidity became evident. The data are recorded in table 4 and show that, the higher the pH at which the acetate extract is shaken, the higher the pH at which acid-precipitable material appears upon adding acid after the shaking. Thus, shaking with chloroform results not only in a new acid precipitation range for the toxin, but in addition removes material which is acid-precipitable at a higher pH than the toxin. That the removal of such material is incomplete in the single shaking is illustrated by the last vertical column of table 4, which records the pH to which the chloroform-shaken acetate extract had to be raised in order to effect complete disappearance of turbidity after its initial appearance resulting from lowering of the pH.

The actual amount of solids removed by two shakings with chloroform was found to be between $\frac{1}{24}$ and $\frac{1}{27}$ of the solids by weight. Therefore, the importance of chloroform shaking in the purification procedure would not seem to lie in the amount of extraneous material removed from solution.

In the case of gelatin solution, the effect of pH and gelatin concentration on the percentage of solids removed from solution into the gel with a single chloroform shaking was determined. For the concentration range from 0.5 to 3.0 per cent there was a greater percentage removal of gelatin at low concentrations than at higher concentrations. In the range from pH 3.5 to 8.5, pH was a minor factor, with a trend toward greater removal at the lower values.

Change in pH of acid precipitation of toxin after chloroform shaking. Soon after studies were started on chloroform shaking, it was observed that, after shaking, the pH of acid precipitation of the toxin from 0.075 M acetate extract had changed from 3.5 to 5. The following experiment firmly established this fact. Sodium acetate (0.075 M) extract of acid mud was shaken twice with one-fourth its volume of freshly distilled chloroform. After shaking and centrifuging, the toxicity of the aqueous phase was 10,000,000 MLD per ml. The mixture was brought successively to the following pH values: 5.8, 5.4, 5.0, 4.6, 4.0, and 3.4. At each pH it was centrifuged and the precipitate redissolved in 0.075 M sodium acetate solution. The toxicity and nitrogen content of the redissolved precipitates are shown in table 5.

TABLE 4

Influence of pH of chloroform shaking on the removal of acid-precipitable material into the gel

SAMPLE	pH AND TURBIDITY BEFORE CHLOROFORM SHAKING	pH AND TURBIDITY AFTER CHLOROFORM SHAKING	AFTER CHLOROFORM SHAKING		
			Acid added	Then alkali added	
			pH of first appearance of turbidity or increase of turbidity	Clearing to control on pH reversal	pH of complete clearing of solution
1	6.95 Not turbid	7.2 None	5.5	above 7*	above 7*
2	6.5 Not turbid	6.62 None	5.4	above 7*	above 7*
3	6.0 Not turbid	6.05 Slight	5.4	6.0	6.8
4	5.5 Slight	5.6 Slight	5.2	5.5	above 7*
5	4.95 Pronounced	5.0 Pronounced	4.2	5.0	5.9
6	4.5 Pronounced	4.57 Pronounced	3.9	5.1	6.6
7	4.02 Pronounced	4.08 Slight	3.4	4.7	5.9
8	3.25 Precipitate formed	3.3 None	2.4	3.4	3.4

* pH above 7 was completely out of the buffer range so that a slight addition of alkali resulted in uncontrolled large changes of pH.

It appears that shaking a sodium acetate extract of toxin with chloroform changes the pH of acid precipitation so that practically complete recovery of toxin is possible at pH 5.0. In terms of nitrogen content this acid precipitation procedure yields material that is five times as pure as that obtained after chloroform shaking. Four successive steps, namely, acid precipitation of culture, extraction of acid mud, chloroform shaking, and precipitation at the new value of pH 5, result in a product which, on the basis of nitrogen content, is more than 50 per cent pure.

Nature of the action of chloroform shaking. It seems desirable to formulate a hypothesis to explain the shift in pH at which toxin is precipitated after chloroform shaking. If we assume that the pH of minimum solubiltiy is the isoelectric point, the change might be supposed to be in the electric charge carried by the toxin molecule. Shaking with chloroform obviously induces a change either in the toxin or in its environment that must account for the new acid range of precipitation. Inasmuch as the toxin remains pharmacologically active and capable of reacting with antibody, the evidence is more for a change in the character of the environment than in the toxin itself. If the toxin were associated in some way (adsorption?) with an extraneous protein, modification of the character of the associated protein by denaturation might well account for the change in acid solubility properties of the toxin. Shaking most proteins with an organic solvent, such as chloroform, would result in denaturation. For extraneous protein to be denatured without affecting the toxin indicates that botulinus toxin has a greater resistance to surface denaturation by chloroform than have most proteins.

TABLE 5

Change in "isoelectric point" of toxin upon shaking with chloroform

TREATMENT	REDISSOLVED PRECIPITATE		MG NITROGEN PER MLD ($\times 10^{-8}$)	% PURIFICATION OF THE STEP IN TERMS OF MG N/MLD
	MLD per ml (millions)	Mg nitrogen per ml		
Chloroform shaking	10	0.67	6.7	
Precipitate at pH				
5.8	<1	Negligible		
5.4	<1	0.01		
5.0	10	0.135	1.35	500
4.6	<1	0.035		
4.0	<1	0.066		
3.4	<1	0.11		
Total nitrogen from precipitates		0.356		
Supernatant at 3.4	0.02	0.307		
Total nitrogen recovered		0.663		
pH 5.0 precipitate purified by salting out at 40% saturation with $(NH_4)_2SO_4$	7.5	0.078	1.04	23
Crystalline toxin			0.83	21

* Acetate extract shaken with chloroform and precipitates collected at various pH values and titrated. Before chloroform shaking the bulk of the toxin is precipitated at pH 3.5.

Our view is that on acid precipitation of the culture, as the pH drops, the toxin becomes associated with extraneous protein. Upon the redissolution of the acid precipitate at pH values above 5.5 (the exact pH will probably vary with the kind and quantity of salt present), the complex dissociates. When shaken with chloroform at this stage, extraneous protein is denatured and a portion removed in the gel, so that when the pH is again lowered association of toxin with extraneous protein does not take place anew. Hence, toxin can be precipitated at a new pH value in its true isoelectric range. Shaking at acid pH values at which the toxin is associated with other proteins results in the active toxin coming down with the denatured protein into the gel. If this working hypothesis is correct, it should be possible to separate the toxin from other proteins by salting out toxin from extracts of acid muds at pH values at which the toxin is not associated with other proteins. Such salted-out toxin, when redissolved in the absence of the extraneous proteins, should then be acid-precipitable in a new pH range. In short, it should be feasible to eliminate chloroform shaking.

This possibility was tested. By repeated precipitation at 20 to 30 per cent saturation with ammonium sulfate, from 0.075 M acetate and 1 M sodium chloride extract of an acid mud at pH 6.5 to 6.8, it was possible to obtain toxin which was acid-precipitable from 0.075 M sodium acetate at pH 5. Before these precipitations material precipitable at 5 per cent saturation was discarded.

Combining the extraction and chloroform-shaking steps into one step. If the hypothesis presented is valid, there is no reason why extraction and chloroform shaking of the acid mud should not be combined into a single step by resuspending the acid precipitate of whole culture in a salt solution and shaking at an appropriate pH value. Tests suggested that the two steps could be combined into one. The concentration of various salts and the nature of the salt influenced the recovery of active toxin in the aqueous supernatant after centrifugation of the gel resulting from chloroform shaking. Thus, 0.7 M NaCl proved superior to 0.5 M $MgSO_4$ at pH 5.5 to 6.5. At pH 6.5 or higher, little recovery of active toxin was obtained, but flocculation tests revealed almost quantitative recovery of material capable of combining with commercial antitoxin.

In one experiment the influence of varying the NaCl concentration at initial pH values of 4.5, 6.0, and 8.0 was tested. The salt was added to 50-ml samples of resuspended acid mud, which were shaken with 12.5 ml of cp chloroform in air for 5 minutes and centrifuged. After the shaking, the pH values had changed from the initial values of 4.5, 6.0, and 8.0 to 4.5, 6.5, and 7.6. As a control, toxin recovery by extraction alone was determined. At pH 4.5 the solution of molar or lower salt concentrations contained more toxin than those with higher concentrations. At pH 6.0 the solutions with salt concentrations below 2 M were of equal toxicities. Although at pH 6.0 the total toxin in solution seemed to be independent of salt concentration, the ratio of toxin to total nitrogen in solution varied with salt concentration. This indicates that salt concentration can be controlled to decrease the amount of extraneous nitrogenous material being brought into solution. Toxin recovery equal to that from extraction alone was possible. Thus combination of the extraction and the chloroform shaking into a single step was included as a part of the purification procedure. At pH 8 recovery of active toxin was poor for all concentrations of salts employed. In further experiments it was determined that the best range for maximum recovery of toxin from an acid precipitate of whole culture, with least total nitrogen in solution, was at a pH adjusted before chloroform shaking in air between 5.0 and 6.0. Flocculation tests on solutions that were shaken at pH 5.0 and 6.5 showed the presence of similar quantities of material capable of combining with antitoxin, though the difference in toxicity was at least a hundredfold. Again it was demonstrated that when chloroform shaking destroys toxicity it does not necessarily destroy antitoxin combining power.

INFLUENCE OF SALTS ON THE ACID PRECIPITATION OF TOXIN

It has been observed repeatedly that acid precipitation of toxin is dependent on salt concentration. This is true of the extract of acid mud both before and immediately after the chloroform-shaking step in the purification procedure. In one experiment a 16-liter culture was precipitated with hydrochloric acid, and a portion of the acid mud extracted with 230 ml of 0.075 M sodium acetate solution at pH 6.5. The extracted toxin was reprecipitated at pH 3.5, and the precipitate resuspended to the original volume in 10 times (0.75 M) the concentration of sodium acetate originally used for extraction. The solution was shaken under CO_2 with 55 ml of cp chloroform for 5 minutes, during which time the pH dropped to 6.4. The 219 ml of toxin solution recovered by centrifugation were slowly acidified. No noticeable precipitate appeared until pH 4.15 was reached. At pH 4.0 the mixture was centrifuged, and the precipitate collected and dissolved in 0.075 M sodium acetate solution at pH 6.6. From this solution the bulk of the toxin precipitated at pH 5. In 0.75 M acetate the toxin precipitated in the pH range of 4.1 to 3.5. Diluting a 0.75 M sodium acetate toxin solution 10 times resulted in toxin precipitation at pH 5. These results were duplicated when toxin solutions, purified beyond the chloroform-shaking step, were used and when sodium chloride and ammonium sulfate were tested.

In short, it appears that increasing the salt concentration of an extract of toxic acid mud from whole culture does not prevent the appearance of a new isoelectric range after chloroform shaking and further purification by the method described. At all stages of purity of the toxin the range for acid precipitation is dependent on salt concentration. Increasing salt concentration seems to spread and lower the pH range of acid precipitation.

CRYSTALLIZATION OF TOXIN

The method of crystallization has been described (Lamanna *et al.*, 1946). It is necessary, however, to emphasize more recent experience which shows that the addition of salts, such as ammonium sulfate, sodium sulfate, and magnesium sulfate, in sufficient quantities to reduce solubility and induce crystallization, though useful, is not essential. By carefully adding at room temperature the smallest amount of 0.075 M sodium acetate adjusted to pH 6.5 that will completely dissolve amorphous toxin and then lowering the temperature, it is possible to obtain crystals almost immediately. In several instances slow evaporation at room temperature has also resulted in crystallization. With some batches of purified toxin, crystals have appeared at room temperature while the amorphous material was going into solution. This phenomenon is probably related to the great difference in solubility that exists between amorphous and crystalline material. The latter fact has also caused experimental difficulties in attaining repeated recrystallization. Irrespective of the manner in which crystals have been obtained they have had the same needlelike shape.

In terms of the number of mouse MLD present in the original mother culture, the yield of crystalline material by the present method has varied from 5 to 20 per cent. It is estimated that no more that 800 milligrams of toxin are present originally in one carboy containing 16 liters of culture; of this, 40 to 160 milligrams can be recovered.

Recrystallization. Recrystallization has been performed in two ways: (a) Crystals are redissolved at room temperature in a minimum amount of distilled water made alkaline (pH 7.5 to 8.5). The solution when placed in the refrigerator yields crystals. Because of the sensitivity of the toxin to alkali this method is probably less preferable than the following. (b) Crystals are suspended in a solution of molar sodium chloride and 0.075 M sodium acetate at pH 4.0. The suspension is dialyzed against the same solvent for several days in the refrigerator. Undissolved material is centrifuged off. The toxin is then salted out with ammonium sulfate at refrigerator temperature. The precipitated toxin is redissolved at room temperature in the least possible amount of 0.075 M sodium acetate at pH 6.5, and crystallization is attempted by any of the methods previously described.

The crystals of the toxin appear to be needle-shaped plates which do not retain their shape on drying. No evidence for birefringence has been found. When crystals are grown on a glass slide by covering a drop of a suspension of crystals with a cover slip and permitting the solvent to evaporate at the edges, the platelike character of the shape is emphasized. The greatest growth appears in width. The kinds of appearances are illustrated in figures 1 and 2.

SOME PROPERTIES OF PURE TOXIN

The pure toxin is a white, odorless, nondialyzable solid. Solubility is a function of temperature, pH, and salt concentration. Dialysis of a dilute salt solution against distilled water is accompanied by a decrease in solubility. Electron microscope pictures of the toxin molecule taken by Robley C. Williams of the University of Michigan, using formalin-treated crystalline toxin, show a single molecular species, nearly spherical in shape, with an estimated size about one-sixth that of the influenza virus, or slightly smaller than the hemocyanin molecule. These data check with estimates of molecular weight based on diffusion coefficient measurements utilizing the membrane diffusion method (Lamanna *et al.*, 1946).

The pure toxin gives a positive reaction in the common qualitative tests for protein. To date the presence of at least 14 amino acids has been detected. All the classes of amino acids are represented. The presence of small amounts of phosphorus (0.045 per cent) and sulfur is indicated. Chemical studies of the nature of the toxin are still in progress, and will be published in the future.

Toxicity. To determine the toxicity of the pure toxin it has been necessary to abandon the MLD as a statement of absolute potency and to substitute an LD50

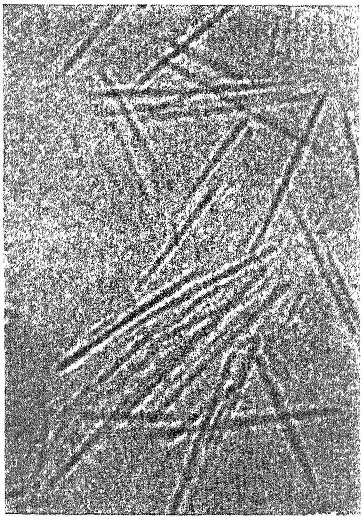

FIG. 1. CRYSTALS OF BOTULINUS TOXIN, TYPE A, ILLUSTRATING THE APPEARANCE OF CRYSTALS COMING OUT OF SOLUTION FROM A SUPERSATURATED SOLUTION OF AMORPHOUS TOXIN. × 450

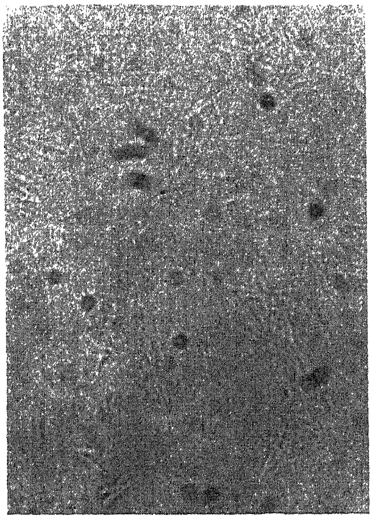

FIG. 2. CRYSTALS OF BOTULINUS TOXIN, TYPE A, GROWN FROM SMALLER ONES BY EVAPORATION OF SOLVENT FROM THE EDGES OF A COVER SLIP PLACED ON A GLASS SLIDE OVER A DROP OF A SUSPENSION OF CRYSTALS. × 450

Note that the ratios of length to width of the crystals pictured in figures 1 and 2 are not the same. Great variation in the size of crystals has been observed for different batches of the toxin. Photos taken by Capt. D. H. Ferris, AUS.

measure based on a statistically valid titration (Bliss, 1935). Amorphous, crystalline, and recrystallized materials have been determined to have the same toxicity within the limits of error of the mouse titration. For the 20-gram white mouse, 0.000032 gamma of toxin equals one LD50 by the intraperitoneal route. This figure is based on the finding that there are 220 million LD50 per milligram of toxin nitrogen, and that the nitrogen content of pure toxin is 14.3 per cent. The latter figure was determined for toxin dried to constant weight in a vacuum desiccator over P_2O_5 after having been dialyzed free of salts. The MLD of the pure toxin is approximately 0.00005 gamma. This figure does not have the statistical validity of that for the LD50.

Before acid precipitation the cultures grown in the 0.3 per cent casein medium contain about 135 million LD50 per gram of dry solids. The potency of pure toxin indicates that the isolation procedure results in about a 240-fold purification.

For the 20-gram white mouse there would appear to be 32 billion intraperitoneal LD50 per gram of dry toxin. This figure makes botulinus toxin the most potent poison known. In comparison with inorganic and simpler organic poisons this is all the more impressive when the actual number of molecules is considered. The toxin has a molecular weight in the neighborhood of a million. Thus a simpler substance of molecular weight 1,000 with a similar potency on a weight basis would actually be 1,000 times less poisonous.

If we assume that the average person weighs 75 kilograms (somewhat on the heavy side), and that man is of the same order of susceptibility as the mouse, only 3,750 times more toxin, or 0.12 gamma, would be required for one LD50. It is, therefore, logical to believe that the reported instances of death resulting from mere tasting of spoiled foods are actual fact. If the foods contained 100,000 mouse MLD per ml, only 0.037 ml would be required to cause death. Food containing 1 million MLD per ml, a not impossible figure, consumed to the extent of 0.0037 ml would be fatal. From these considerations it is easy to understand that, even if absorption from the alimentary tract were extremely poor, ingestion of amazingly small quantities of contaminated food might result in toxemia.

The small quantities of toxin required for poisoning lend importance to investigations designed to gain information about the structure and configuration of the molecule that may explain the extreme toxicity. Such studies have been initiated.[5] Denaturation accompanied by loss in solubility invariably leads to detoxification. This implicates the molecule as a whole in playing a role in toxicity. The pure toxin is readily detoxified by surface denaturation. This is an interesting observation because under specified conditions the impure toxin can be shaken vigorously with chloroform with impunity. The toxin is sensitive to alkali. In this connection the presence of fairly large quantities of *beta*-hydroxyamino acids (about 11 per cent) may have significance. A dissimilar array of chemical substances can cause detoxification. Among these are copper sulfate, phenol, thymol, hydroquinone, ketene, and ethylene. Some incomplete evidence has been obtained which indicates partial detoxification by carbon monoxide.

[5] E. J. Schantz, H. J. Buehler, and D. H. Bornor are participating in these studies.

SUMMARY AND CONCULSIONS

A method for purifying and crystallizing type A botulinus toxin has been discussed.

Extraction of the toxin from an acid precipitate of whole culture is determined by the pH of the extracting solvent. The efficiency of extraction and the stability of the toxin are influenced by the concentrations of salts present. Shaking an extract of acid precipitate of culture with chloroform results in the formation of a protein gel. Depending on the pH and concentrations of salts present, the toxin is detoxified, or remains active and in solution, or remains active and composes a part of the gel. Shaking with chloroform at a high pH value (6.8+) results in detoxification with no equivalent loss in antitoxin combining power. During the shaking the pH changes, the direction and the extent of the change depending on the nature of the buffers present and the salt concentration. After being shaken with chloroform, toxin is recovered which is acid-precipitable in a new isoelectric range. A hypothesis to explain this finding is discussed.

Increasing the salt concentration seems to spread and lower the pH range of acid precipitation of the toxin at all stages of purity.

The pure toxin is a protein with the solubility properties of a globulin. An LD50 for the 20-gram white mouse contains 4.5×10^{-9} mg of nitrogen; there are about 32 billion intraperitoneal LD50 per gram of dry toxin. On the basis of dry weight, the pure product is approximately 240 times more potent than the toxin in the original mother culture.

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THE TAXONOMIC POSITION OF CORYNEBACTERIUM ACNES

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The organism known as the "acne bacillus" in medical literature was first observed by Unna (1896) in histological sections of acne comedones, but Sabouraud (1897) was the first to cultivate this organism successfully in pure culture from the contents of acne pustules. Subsequent studies on the etiology of acne vulgaris by Gilchrist (1900, 1903), Hallé and Civatte (1907), Hartwell and Streeter (1909), Fleming (1909), Südmerson and Thompson (1909), and Molesworth (1910) indicated that the acne bacilli were morphologically similar to the corynebacteria but differed markedly from these organisms in showing a strong preference for anaerobic conditions. Gilchrist (1900) named the organism *Bacillus acnes*, whereas Bergey *et al.* (1923) placed it in the genus *Corynebacterium* because of its morphological relationship to the members of this group.

Our interest in these organisms became aroused following the observation of Weiser and Gunter (1942) of this laboratory that samples of human blood plasma destined for a civilian plasma bank contained anaerobic diphtheroids as one of the principal contaminants. These workers were able to show that such organisms occurred on normal skin and probably gained entry into the blood plasma as the result of ineffective skin disinfection preceding venipuncture.

The anaerobic diphtheroids isolated from plasma and skin seemed to fit the general description of *Corynebacterium acnes* (Bergey *et al.*, 1939), but, as the inclusion of such organisms in the genus *Corynebacterium* seemed questionable to us, we considered it worth while to make a comparative study of a series of isolates and the available authentic cultures of *C. acnes* in an attempt to clarify their taxonomic position. It also seemed desirable to obtain quantitative data on the occurrence of these organisms on normal human skin.

CULTURES EMPLOYED

Cultures of *C. acnes*, numbers 6919, 6920, 6921, and 6923, were obtained from the American Type Culture Collection. These cultures were originally obtained from the National Collection of Type Cultures, England, and had been isolated from clinical cases of acne vulgaris. In addition, 27 cultures of anaerobic diphtheroids were isolated from the skin of ten normal subjects, 4 cultures from different samples of human blood plasma, and 1 culture from a clinical case of acne vulgaris.

CULTURE MEDIUM

The medium recommended by the U. S. Office of Civilian Defense for sterility testing of blood plasma supports a fair growth of all strains, but because of acid production the pH soon becomes limiting in this poorly buffered medium and growth ceases. A highly buffered modification of this medium supports a luxurious growth of all strains and is recommended for isolation and routine culture work. The modified medium has the following composition: bacto peptone, 2 per cent; bacto yeast extract, 0.5 per cent; glucose, 1.0 per cent; KH_2PO_4, 2 per cent; B. B. L. sodium thioglycolate, 0.10 per cent; and agar, 0.05 per cent, or 1.5 per cent, depending upon whether a fluid or solid medium is desired. The pH should be adjusted to about 7.1 before sterilization and will be about 6.8 after sterilization. Considerable darkening of the medium takes place during autoclaving, but this has no apparent adverse effect upon the growth of the organisms.

ISOLATION PROCEDURE AND OCCURRENCE ON NORMAL SKIN

The isolation of cultures from normal skin and the estimation of the relative numbers of these organisms on skin were accomplished as follows: An area of about 2 square inches of skin on the upper arm was scraped with a sterile Bard-Parker blade moistened in sterile saline, and the skin scrapings were transferred to a sterile mortar containing 5 ml of sterile saline and some fine sand. After thorough maceration, dilutions of the resulting suspensions were plated, using the medium described above, and incubated under hydrogen in a McIntosh-Fildes jar for 4 days at 37 C. Plates which showed well-separated colonies were counted, and all of the colonies from the counted plates were streaked and stabbed on deep butt agar slants of the same composition as the plating medium. After 4 days' incubation at 37 C, all of the resulting transfers were examined by gram staining and inspected for growth in the stab and on the slant. Anaerobic diphtheroids were readily recognized by their morphology and by the fact that growth occurred only in the stab and never on the slant. The flora of the skin of 7 individuals was examined in this way, and the occurrence of anaerobic diphtheroids, expressed as a percentage of the total skin flora capable of developing under anaerobic conditions, is tabulated in table 1. As anaerobic diphtheroids accounted for from 9 to 78 per cent of the total skin flora capable of growth under these conditions, it is obvious that organisms of this type constitute a significant part of the normal skin flora. The remainder of the flora of the subjects examined by us consisted almost entirely of nonpigmented facultatively anaerobic micrococci.

TABLE 1

The occurrence of anaerobic diphtheroids on normal skin

SUBJECT	NUMBER OF COLONIES EXAMINED	% ANAEROBIC DIPHTHEROIDS
E. F.	45	40
J. F.	28	14
V. E.	43	9
E. J.	33	33
R. W.	48	50
G. D.	27	78
P. M.	48	10

The occurrence of anaerobic diphtheroids on normal individuals was first noted by Lovejoy and Hastings (1911), who found these organisms to occur in large numbers and in almost pure cultures in the sebum expressed from the sebaceous glands around the folds of the nose. We have made no isolations from this source, but the examination of gram stains of such material has served to verify the observations of those workers. In all probability the normal habitat of these organisms is the sebaceous glands of the skin, and in areas such as those around the nose where the glands are large and very active in secreting sebaceous material the organisms occur in great abundance.

DESCRIPTION OF STRAINS

Relation to temperature and oxygen. The optimum temperature for growth is approximately 37 C, and at this temperature the maximum crop is reached in liquid media in 3 to 4 days. No growth occurs at 45 C, and growth at room temperature is extremely slow.

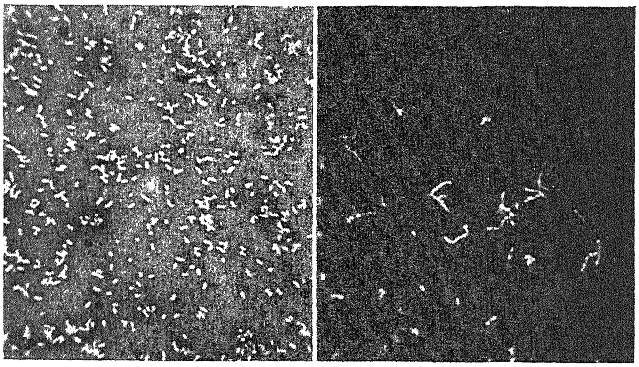

FIG. 1. STRAIN 22. NEGATIVE STAINS OF FOUR-DAY-OLD SLANT CULTURES INCUBATED (a) ANAEROBICALLY AND (b) AEROBICALLY. × 970

All strains show a marked preference for anaerobic conditions, although growth consisting of a few isolated colonies can usually be obtained on slant cultures exposed to the air if a heavy inoculum is used. Six strains, however, have consistently failed to grow aerobically, but on the other hand one strain has been found to grow almost as well aerobically as anaerobically. Apparently a wide range of oxygen tolerance occurs within this group, but there appears to be no correlation between the relation to oxygen and other characteristics.

Morphology. All strains, when examined from fluid or anaerobic slant cultures, present a similar morphological picture, the organisms ranging from small, plump rods to ellipsoids which tend to occur in pairs with the cells joined at a slight angle (figure 1a). The size of the individual cells in gram stains from such cultures is approximately 0.4 to 0.5 by 0.8 to 0.9 microns.

Cells from aerobic slant cultures appear to be somewhat longer and more pleomorphic than those in anaerobic cultures, but the differences in morphology under these two conditions are, in most cases, slight. An occasional strain, however, will show marked differences in morphology between aerobic and anaerobic cultures, cells from aerobic cultures being much longer and swollen or club-shaped, and sometimes showing what seems to be rudimentary branching (figure 1b). Such morphology is somewhat similar to that found normally in the corynebacteria and has previously been reported for *C. acnes* by Gilchrist (1903) and Südmerson and Thompson (1909). This effect of oxyen on the morphology is very much like that described by van Niel (1928) for the propionic acid bacteria which show marked pleomorphism in aerobic cultures.

All strains are strongly gram-positive. Endospores are not formed, and motility is absent.

Appearance of growth on solid and liquid media. Surface colonies on streaked plates after 4 to 5 days of anaerobic incubation are circular, raised, smooth, glistening, and have an entire edge. The size varies from 1.5 to 4.0 mm in diameter. The color of the colonies is quite characteristic, for after 4 to 5 days of incubation there is a faint but definite tinge of pink, which intensifies upon further incubation to a pale salmon pink.

Liquid cultures containing 0.05 per cent agar tend to develop at first as small, discrete colonies, especially if started from small inocula, but this phenomenon tends to become obscured upon further growth of the culture. The sedimented cells of such cultures appear cream-colored at first, but, like colonies on agar plates, become a pale salmon pink upon further incubation.

Biochemical Reactions

Utilization of carbon compounds. As the examination of glucose broth cultures revealed that large amounts of volatile acid are produced, acid production was used as a criterion of the utilization of various carbon compounds. Volatile acid production, which in a highly buffered medium containing 1.0 per cent glucose frequently equaled 10 ml of M/10 acid per 10 ml of medium, was determined by direct titration of the cultures, by titration of steam distillates, or by notation of the color change of an indicator incorporated into the medium. As the results obtained were found to be qualitatively the same regardless of the method used, the latter procedure was chosen, except in the case of lactate, because of its simplicity. The utilization of lactate was determined by visual comparison of the growth in the basal medium alone and in the basal medium containing lactate, and in several cases by volatile acid determinations.

The basal medium used for determining acid production by the indicator method contained 2.0 per cent peptone, 0.5 per cent yeast extract, 0.5 per cent of the carbon compound, 0.1 per cent sodium thioglycolate, 0.05 per cent agar, and Andrade's indicator. The medium was sterilized in 10-ml amounts in tubes containing small inverted vials for gas traps. Incubation was in air at 37 C for 7 days. The results are summarized in table 2. It can be seen that several compounds are fermented with the production of acid but no visible gas, and although the organisms can be separated into 4 groups on the basis of the carbon compounds fermented, we have been unable to correlate these fermentation groups with the sources of the cultures, or other characteristics, and therefore attach no taxonomic significance to them.

Liquefaction of gelatin. Gelatin is liquefied in 10 days at 37 C by all strains in peptone yeast-extract phosphate glucose gelatin "deeps."

Litmus milk. Standard litmus milk was exhausted by steaming, cooled, inoculated, and layered with sterile vaspar. All strains produced a rennet curd after about 2 weeks' incubation at 37 C, followed by a slow peptonization of the casein.

Reduction of nitrates. All strains reduce nitrates to nitrites in peptone yeast-extract phosphate glucose thioglycolate broth containing 0.2 per cent KNO_3.

Production of indole. About one-half of the strains produce indole in tryptose yeast-extract phosphate glucose thioglycolate broth.

Catalase reaction. Strongly positive for all strains.

Hemolysis. All strains produce *beta*-hemolysis on peptone yeast-extract glucose phosphate agar containing 5.0 per cent by volume of citrated human blood.

TABLE 2

The utilization of carbon compounds

GROUP	NUMBER OF CULTURES	GLUCOSE	MANNOSE	GALACTOSE	FRUCTOSE	GLYCEROL	MANNITOL	MALTOSE
I	27	+	+	+	+	+	−	−
II	6	+	+	+	+	+	+	−
III	3	+	+	+	+	−	−	−
IV	1	+	+	−	+	+	−	+

None of the cultures utilized lactate, arabinose, sucrose, lactose, raffinose, salicin, inulin, or starch.

+ = acid production but no visible gas.

Although the 37 strains show certain differences in their cultural characteristics, we can see no logical basis for their separation into more than one group and consequently consider them closely related, if not identical. It thus seems justifiable to conclude that the strains isolated from the skin, blood plasma, and cases of acne vulgaris belong to a single species that is now known as *Corynebacterium acnes*. The question of the inclusion of this organism in the genus *Corynebacterium*, however, will be discussed after a consideration of the nature of its catabolic process.

NATURE OF THE FERMENTATION OF GLUCOSE

Nothing has been reported concerning the nature of the fermentation produced by *Corynebacterium acnes* beyond the routine observation by several workers that acid but not gas is produced from certain carbohydrates. It is evident, however, from the fact that only a negligible amount of growth takes place in the absence of sugars that sugar fermentation represents the principal mode of energy liberation for this organism. The significance of the nature of the fermentation in the classification of facultative and anaerobic bacteria has been emphasized by Kluyver and van Niel (1936) and Barker and Haas (1944), although this important point is frequently not appreciated by bacteriologists.

The general morphological picture, the relation to oxygen, and the fact that volatile acids are produced in the fermentation of glucose suggested a close relationship between *Corynebacterium acnes* and the propionic acid bacteria. To identify the volatile acids produced by *C. acnes* analyses were made by means of the Werkman (1931) partition method using the systems ethyl ether and water and isopropyl ether and water. The results indicated the presence of propionic and acetic acids in the ratio of 1.7 to 2.2 parts of propionic to 1 part of acetic acid. The presence of propionate in concentrated solutions of the sodium salts of the volatile acids was verified microchemically by the characteristic appearance of the mercurous salt (Klein and Wenzl, 1932), but attempts to demonstrate acetate by means of the mercurous salt or by the formation of sodium uranyl acetate (Klein, 1932) failed because of interference by the propionate. In order to demonstrate conclusively the presence of acetate, the acetic and propionic acids were separated by the azeotropic distillation procedure of Schicktanz *et al.* (1940). After this had been accomplished, acetate was easily detected by either of the microchemical reactions just mentioned.

TABLE 3

The fermentation of glucose by Corynebacterium acnes, strain A.T.C.C. 6920

	mM	MILLI-ATOMS CARBON	MILLI-EQUIVALENTS OF AVAILABLE HYDROGEN[*]
Glucose	−11.20[†]	67.20	268.8
CO_2	+7.10	7.10	0
Acetic acid	+8.15	16.30	65.2
Propionic acid	+13.85	41.45	191.2
Total recovery		64.85	256.4
% Recovery		96.5	95.3
O/R index		1.02	

[*] Calculated according to the method of Barker (1936).

[†] (−) = product used; (+) = produced produced.

That fixed acids are not formed in appreciable amounts in the fermentation of glucose was demonstrated by the fact that practically all of the acid produced could be accounted for in the acids volatile in steam. In some cases a small amount of nonvolatile acid is apparently produced, but because of the amounts involved its identity has not been determined.

Neutral volatile products were shown to be absent by applying the usual microchemical tests to distillates obtained from neutralized cultures.

Although no visible gas is formed in ordinary cultures, CO_2 formation could be demonstrated either by culturing in Eldredge tubes, or by collecting the gases over mercury from 500 ml of culture contained in an all-glass fermentation vessel.

The gas produced in the latter procedure was shown to be pure CO_2 by the fact that it was completely absorbed by 5 per cent KOH.

Although the qualitative analyses indicated that *Corynebacterium acnes* produces a propionic acid fermentation of glucose, it was desirable to verify this conclusion by means of quantitative experiments. This was accomplished by analyses of cultures grown in large Eldredge tubes containing 200 ml of 0.18 N $Ba(OH)_2$ in one compartment for the absorption of CO_2, and 250 ml of culture medium containing 1.0 per cent glucose in the second compartment. Because of the interference of sodium thioglycolate in the determination of reducing sugars, it was necessary to omit this substance from the medium, otherwise the medium was as previously described. Incubation was at 37 C under an atmosphere of oxygen-free nitrogen for a period of 10 days, after which time the fermented medium and an uninoculated control were analyzed. The results of such an experiment, using *C. acnes* A.T.C.C. no. 6920, are presented in table 3 and demonstrate clearly that this organism produces a propionic acid fermentation, as practically all of the carbon and available hydrogen in the glucose fermented can be accounted for in carbon dioxide, propionic acid, and acetic acid. Less extensive work with the remainder of the cultures indicates that all strains produce the same type of fermentation of glucose.

DISCUSSION

C. acnes and a second anaerobic diphtheroid, *C. lymphophilum*, were placed in the genus *Corynebacterium* by Bergey *et al.* (1923) purely on the basis of morphological considerations. *C. lymphophilum* was isolated by Torrey (1916) from abnormal lymph glands, and although the original cultures are no longer available for study, it seems highly probable from Torrey's description of this organism that it is identical with *C. acnes*.

On a morphological basis *C. acnes* might be placed in either the genus *Corynebacterium* or the genus *Propionibacterium*, for these two groups have certain morphological features in common. Two facts, however, indicate that *C. acnes* is much closer to the propionic acid bacteria than to the corynebacteria. First, the nature of the catabolic process clearly relates *C. acnes* to the propionic acid bacteria. It is true that Tasman and Brandwijk (1938) found propionic acid to occur as a product of sugar dissimilation by *C. diphtheriae*. In the Tomcsik strain studied by these workers propionic acid accounted for 30.3 to 49.9 per cent of the glucose fermented, and in the Bandoeng strain, 5.9 to 6.6 per cent. The fermentations studied by Tasman and Brandwijk were conducted under highly aerobic conditions, however, and may not be representative of the catabolic process of these organisms under strictly anaerobic conditions. Fujita and Kodama (1934) and Friedemann (1940), on the other hand, did not report the presence of propionic acid in the anaerobic fermentation of glucose by *C. diphtheriae*, but indicated that the principal dissimilation products produced were lactic, acetic, formic, and succinic acids, and ethanol. Although it is somewhat difficult to reconcile the conflicting results of these workers, it seems reasonable to conclude that the typical mode of sugar fermentation by *C.*

diphtheriae is not a propionic acid fermentation, although propionic acid may be formed under certain conditions or by certain strains.

Secondly, the effect of oxygen in either completely or strongly inhibiting growth of *C. acnes* markedly distinguishes this organism from the typically aerobic corynebacteria and relates it to the propionic acid bacteria, which also show a decided preference for anaerobic conditions.

In view of the foregoing considerations we feel that *C. acnes* shows far stronger affinities to the genus *Propionibacterium* than to the genus *Corynebacterium*, and suggest, therefore, that this organism be transferred to the former genus as *Propionibacterium acnes* (Gilchrist) Douglas and Gunter *comb. nov.* Such a transfer would require a modification of the diagnosis of the genus *Propionibacterium* (Bergey *et al.*, 1939) to exclude the phrase "ferments lactic acid."

The strains of *P. acnes* studied here seem to form a homogeneous group that can be differentiated from other species of *Propionibacterium* on the following bases:

(1) *Optimum temperature.* Previously described species of *Propionibacterium* have an optimum temperature of 30 C, whereas *P. acnes* has an optimum of 37 C.

(2) *Gelatin liquefaction, action on milk, and nitrate reduction.* None of the previously described species of *Propionibacterium* liquefy gelatin or digest milk, and only one species, *P. pentosaceum*, reduces nitrates.

(3) *Lactate fermentation.* All previously described species of *Propionibacterium* ferment lactate, but *P. acnes* does not ferment this substance.

(4) *Habitat.* To the best of our knowledge, *P. acnes* has been isolated only from man, whereas practically all other species of *Propionibacterium* have been isolated from dairy products.

SUMMARY

The organism previously known as *Corynebacterium acnes* is transferred to the genus *Propionibacterium*, as *P. acnes*, on the basis of its relationship to oxygen and the nature of its catabolic process.

P. acnes occurs on normal human skin, where it constitutes a significant part of the skin flora.

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THE SEPARATION OF DIFFERENT STRAINS OF BACTERIOPHAGE FROM A CRUDE CULTURE

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The bacteriophages studied in the present investigation were obtained directly from the soil by means of a strain of pea *Rhizobium* P_{11}. The crude culture of the phage, which was designated S_2P_{11}, has been the subject of two previous papers (Kleczkowska, 1945*a*, 1945*b*). This phage, when supplied to different strains of *Rhizobium*, produced plaques on susceptible bacteria that differed in mean numbers and size according to the bacterial strain used. This suggested that phage S_2P_{11} might represent a mixture of different strains of phage. This paper describes several such strains isolated from phage S_2P_{11} by means of successive passages through different susceptible bacterial strains and by cultivation from a single plaque.

MATERIAL AND METHODS

The method of obtaining bacteriophage S_2P_{11}, of its cultivation on liquid medium or on agar plates (using the poured plate technique), and also the origin of bacterial strains are described in the preceding papers (Kleczkowska, 1945*a*, 1945*b*).

Antisera to different phage cultures were produced by 6 intravenous injections into rabbits of 1.5 ml of liquid phage culture. The animals were injected twice a week and bled 8 to 10 days after the last injection. Five-tenths per cent of phenol was added to the sera to prevent development of any bacterial contamination.

Inhibition tests were made by adding antisera at different dilutions to samples of phage cultures diluted 1:100 with sterile medium. The mixtures were incubated for 1 hour at 25 C and added in a ratio of 1:10 to 24-hour liquid bacterial cultures. These were either incubated for 48 hours at 25 C, after which the absence of lysis was considered as an evidence of inhibition of lytic activity of the phage, or they were plated as described previously and incubated for 48 hours at 25 C, the plaques then being counted. Total absence of plaques, or a large decrease in their numbers, indicated an inhibiting influence of the antiserum.

RESULTS

Phage S_2P_{11}, as originally isolated from soil on the strain of pea nodule bacteria P_{11}, develops plaques of very unequal sizes. This inequality is brought out more clearly when the percentage of agar used in the plating medium is reduced. The frequency distributions of plaque diameters obtained with phage S_2P_{11} on a range of agar concentrations are shown graphically in figure 1.

An attempt was made to separate the large from the small plaque phage by isolation from single plaques. A small and a large plaque were cut out, and

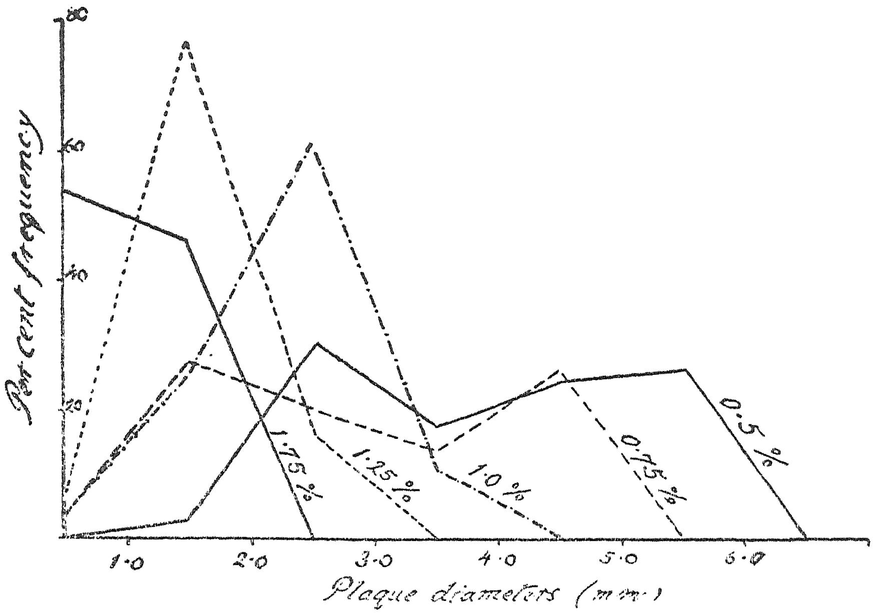

FIG. 1. PERCENTAGE FREQUENCIES OF PLAQUE SIZE PRODUCED BY PHAGE S_2P_{11} IN DIFFERENT AGAR CONCENTRATIONS

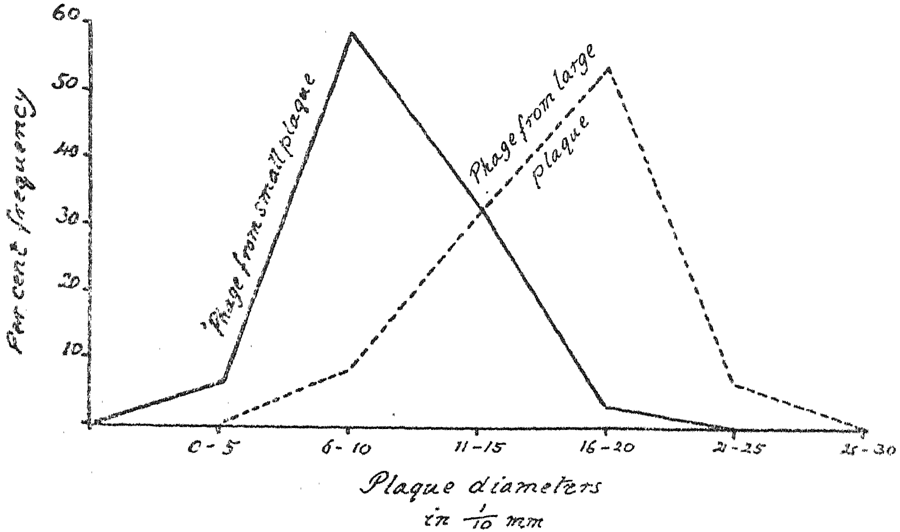

FIG. 2. FREQUENCIES OF PLAQUE DIAMETERS OF PHAGE ISOLATED FROM A LARGE AND FROM A SMALL PLAQUE ON A PLATING OF PHAGE S_2P_{11}

each was put into a 24-hour liquid culture of P_{11} bacteria. After 48 hours of incubation, when both cultures became clear, platings were made and the resulting plaques measured. The frequencies of plaque sizes, shown in figure 2, indicated that some separation had been effected. The two cleared cultures, from which this plating had been made, were subcultured in fresh 24-hour

TABLE 1

Relative plaque numbers produced by different phage strains on different strains of host bacteria

NAME OF THE BACTERIAL STRAIN	NAME OF THE PHAGE STRAINS				
	S_2P_{112} large plaque	S_2Cl_3	$S_2$20272	$S_2$317	S_2P_{11} crude
P_{11}	100	125	135	120	100
Cl_3	none	100	57	none	<0.1
20272	none	73	100	none	<0.1
317	not tested	none	none	100	15

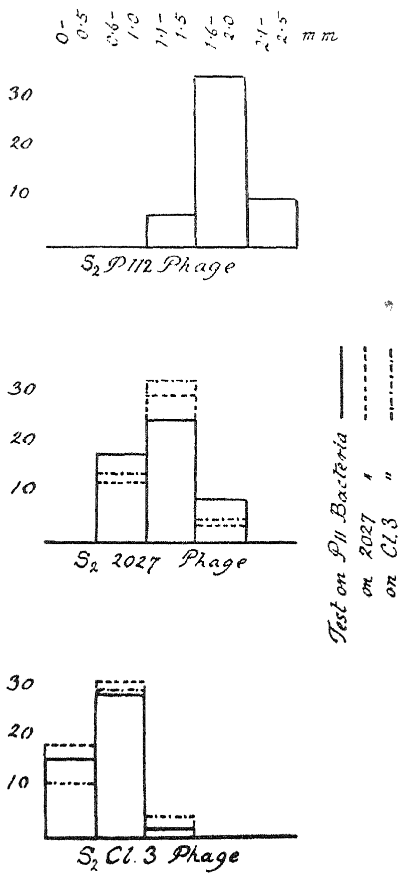

FIG. 3. FREQUENCIES OF PLAQUE DIAMETERS OF THREE PHAGE STRAINS

bacterial cultures, and a second set of platings were made. Then, however, identical plaque size distributions, characteristic of the small plaque phage, were obtained. This illustrates the difficulty of isolating the large plaque phage, owing to the likelihood that the large plaque will contain some small plaque phage. It is, however, possible to obtain strains of phage giving only large and only small plaques, respectively, by repeated isolations from plaques, combined with the use of two bacterial hosts having different susceptibilities. This was done in the following experiment.

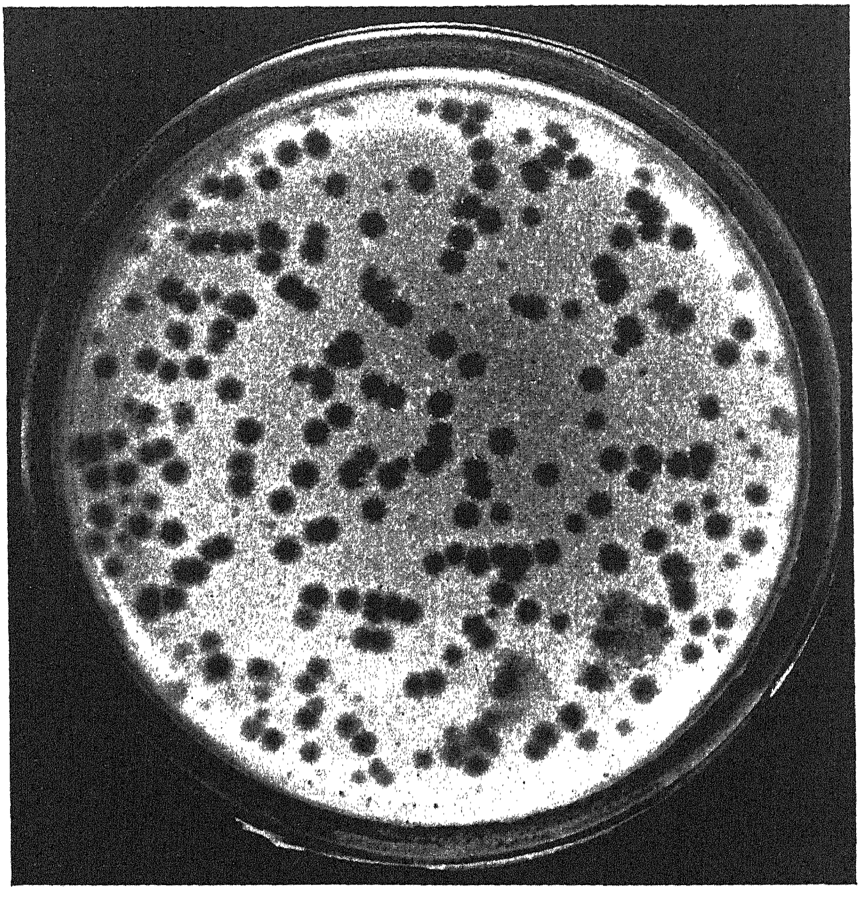

FIG. 4. S_2P112 LARGE PLAQUE PHAGE

Two large plaques were cut from a plating of phage S_2P_{11} on P_{11} bacteria. One of these was placed in a 24-hour liquid culture of P_{11} bacteria and the other in the similar culture of clover nodule bacteria, strain Cl_3. After 48 hours of incubation, each culture was plated on both strains of host bacteria. The phage from the P_{11} liquid culture produced a mixture of large and small plaques when plated on P_{11} bacteria, but produced only small plaques on Cl_3 bacteria. The phage from the Cl_3 liquid culture produced only small plaques on both host bacteria. By several successive passages through P_{11} bacterial cultures, with isolations from large plaques every time, a phage strain was obtained that produced only large plaques on P_{11} and that would not grow on Cl_3 bacteria. By a similar process and with Cl_3 as the host bacteria, a strain of phage was isolated that produced only small plaques on both P_{11} and Cl_3 bacteria.

By similar methods using strains 20272 of clover bacteria and strain 317 of pea bacteria, two other strains of phage were separated from the crude phage S_2P_{11}. A comparison of all four isolated phage strains is given in table 1, which shows the relative number of plaques developed by suspensions of each phage plated on different bacterial hosts, in percentage of the plaque numbers formed with the organism used to isolate the strain of phage.

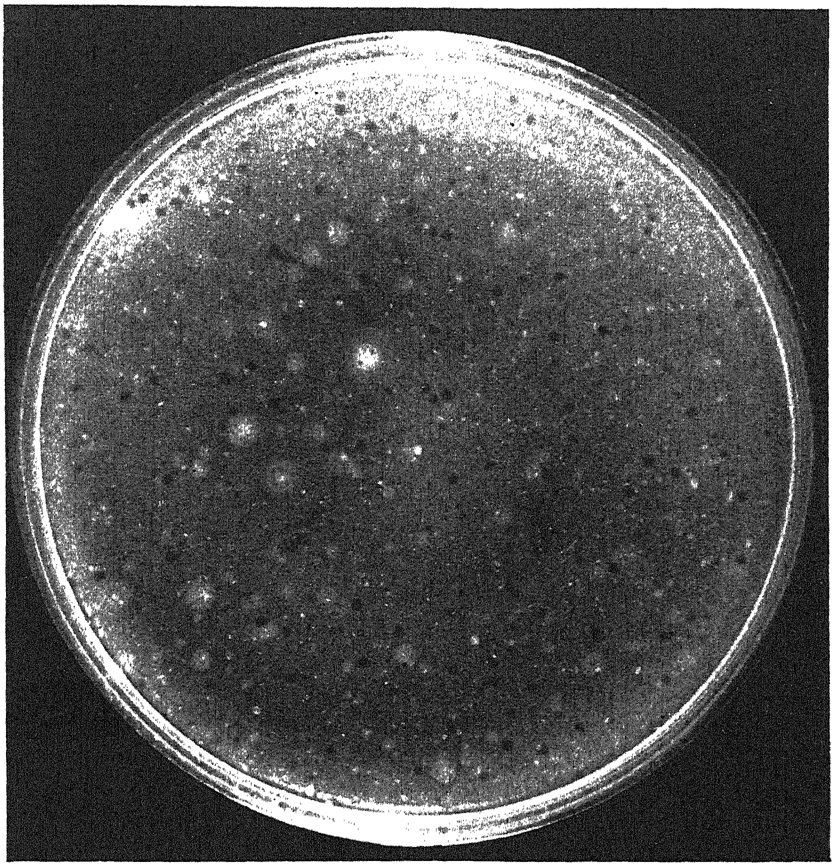

FIG. 5. S_2Cl_3 SMALL PLAQUE PHAGE

The four strains of phage also differ characteristically in their plaque sizes. The histograms in figure 3 show the percentage frequencies of plaque diameters of three of these phage strains plated on each of the three bacterial strains used to separate them. Each strain of phage has a characteristic size of plaque (figures 4 and 5).

Three of the separated phage strains have been found difficult to maintain in culture. This is particularly the case with the large plaque strain which was separated on P_{11} bacteria. It has not been found possible to keep this strain for more than a few transfers to fresh bacterial cultures. The phage strain developed on bacterial strain 20272 died out after a few weeks. However, that developed on bacterial strain Cl_3 was kept for about a year before it died out.

A second strain of phage was developed by culture of S_2P_{11} phage on Cl_3 bacteria. This differed from the first strain isolated on this host by growing very poorly on the Cl_3 bacteria and by producing still more minute plaques on them. It was found, however, that this phage strain grew well on clover nodule bacteria of strain A and on the pea strain 317, forming abundant plaques on these bacteria. This phage strain, which was named S_2A, has been found to be easily maintained in the laboratory. It produced very minute plaques in 1 per cent agar, which were difficult to count and measure. Plaques of measurable size were, however, produced in 0.7 per cent agar, and their mean diameter on different host bacteria is shown in table 2. This phage produced plaques of different sizes according to the bacterial hosts.

TABLE 2

Characteristic plaque sizes of phage S_2A for different bacterial strains

	NAME OF THE BACTERIAL STRAINS			
	A	Clover 3	P_{11}	317
Diameter of the plaques of phage S_2A in mm.	1.2	0.5	1.8	1.6

The separation of phage strains S_2P_{112} (large plaque), S_2Cl_3, S_220272, S_2317, and S_2A from the crude S_2P_{11} phage resembles a mere subdivision of an original mixture of strains by selection on the different host bacteria. After separation they all retained the power of attacking the original host bacteria, strain P_{11}.

Burnet *et al.* (1937), working with coli-dysentery phages, found it possible to classify these into a small number of serological types, but, even among the members of one type, phages completely identical serologically were scarcely ever found. It seemed interesting to discover whether the strains separated from a single crude phage differed from each other serologically.

The phage S_2P_{11} and the two phage strains S_2317 and S_2A isolated from S_2P_{11} were tested serologically with antisera produced against each of them. The results, given in table 3, show that, although the three phage cultures were serologically related, they were not identical. Comparison of strains S_2317 and S_2A shows that each of them was inhibited by a higher dilution of its own antiserum than of the antiserum against the other strain. The crude phage S_2P_{11} contained all antigenic characters present in both S_2317 and S_2A phages, and in addition showed the presence of other characters. This can be concluded from the fact that, whereas its antiserum was highly effective in inhibiting its own lytic activity and that of the two other phage cultures (even with higher dilution end points than their own antisera), antisera against the other two inhibited its activity at a much lower dilution end point than its own antiserum. It must be emphasized that only the effects of different antisera on the same phage can be compared directly. Effects of the same serum on different phages cannot be compared directly, as every phage was tested with its corresponding bacterial strain, so that interactions of different phage bacterial systems represented an additional series of variables.

Normal rabbit serum, or antisera to other antigens, including antisera against *Rhizobium* strains P_{11} and A, tobacco mosaic virus, and human albumin, were all without effect on the number of plaques, neither had they any inhibiting effect on lysis in liquid cultures, even at a dilution of 1:10, although at this dilution homologous phage antiserum completely inhibited phage activity. In this respect the behavior of phage differs from that of plant viruses, whose infectivity is more or less inhibited unspecifically by the presence of almost every protein, so that the effect of the homologous antiserum differs only quantitatively from that of a heterologous antiserum or of a normal serum (Kassanis, 1943). No precipitation could be observed in phage cultures mixed with the antiserum over a range of ratios and then incubated either at room temperature or at 40 C, even when there was complete inhibition of lysis. It is possible that the phage concentration was too low for precipitation to appear.

TABLE 3

Inhibition of lytic activity of three phage cultures by antisera prepared against each of them

NAME OF THE PHAGE CULTURES	ANTISERUM AGAINST	ANTISERUM DILUTIONS					
		100	500	1,000	2,000	3,000	4,000
S_2P_{11}	S_2P_{11}	+	+	+	+	−	−
	S_2317	+	−	−	−	−	−
	S_2A	+	−	−	−	−	−
S_2317	S_2P_{11}	+	+	+	+	−	−
	S_2317	+	+	+	−	−	−
	S_2A	+	−	−	−	−	−
S_2A	S_2P_{11}	+	+	+	+	+	−
	S_2317	+	+	+	−	−	−
	S_2A	+	+	+	+	−	−

+ indicates inhibition (no lysis).
− indicates no inhibition (lysis).

In order to test whether an inactive phage-antiserum mixture can be reactivated by dilution well beyond the limit of the inhibiting activity of the serum, the antiserum was mixed with an undiluted liquid culture of phage S_2P_{11} in a ratio 1:10, and after 1 hour of incubation at 25 C the mixture was added to a 24-hour liquid culture of P_{11} bacteria at dilutions varying from 1:10 to

1:10⁶ and plated. Control plates were also poured from dilutions of phage without antiserum. In these plaques were formed in decreasing numbers with increasing phage dilutions, whereas no plaques were formed on plates with the diluted phage-antiserum mixture. This shows that the phage, when inactivated by a sufficient concentration of a specific antiserum, remains inactive after dilution well above the limit of activity of the antiserum added to it. In this again, phage differs from plant viruses, which, after inactivation by antiserum, regain their activity on dilution beyond this limit (Kassanis, 1943). This is in general agreement with the findings of Burnet *et al.* (1937).

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SUMMARY

A number of different strains of phage were separated from a crude phage S_2P_{11} which was isolated directly from the soil by means of a strain of pea nodule bacteria.

The strains of phage differed in host specificity and in the size of plaques produced by them.

Serologically they were related but not identical.

Lytic activity of the phage after inhibition by its antiserum could not be regained by dilution beyond the limits of serum activity.

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IN VITRO STUDIES CONCERNING THE ACTION OF PENICILLIN ON THE VIRIDANS STREPTOCOCCI, INCLUDING OBSERVATIONS ON THE SO-CALLED SYNERGISTIC EFFECT OF SULFONAMIDE DRUGS

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This investigation was stimulated by our interest in the treatment of subacute bacterial endocarditis with penicillin. We were anxious to learn something of the mode of action of penicillin on the viridans streptococci, the more common causative agents of this disease, but, after reviewing the relevant medical literature, we were left confused by conflicting opinions and seemingly contradictory statements. Therefore for purposes of clarification certain *in vitro* experiments were conducted and are reported herewith. The particular object of these studies concerning the mode of action of penicillin was the determination of the effects, if any, of (1) variations in the number of organisms exposed, (2) variations in the concentration of penicillin, and (3) the presence of sulfonamide drugs.

MATERIALS AND METHODS

Broth. All broth used in the experiments described in this report is the same as that used for many years at the House of the Good Samaritan for the preparation of streptolysin. It is essentially the same as the beef heart infusion described by Swift and Hodge (1933) except that the buffer has been modified so as to contain 3 grams of glucose instead of 2 grams, and 2 grams of $Na_2HPO_4 \cdot 12\ H_20$ instead of 1 gram, for every liter of infusion (Mote, unpublished).

Organisms. All organisms used for tests in these experiments have been strains of viridans streptococci isolated from the blood of patients ill with subacute bacterial endocarditis. Some of the strains were kindly supplied by Dr. Maxwell Finland of the Boston City Hospital. Most of the organisms have been subcultured several times or more in the course of the studies; others have been kept in the frozen and dried state until needed for the tests. In all experiments a freshly made overnight subculture in broth has been used. All dilutions, as indicated in each experiment, have likewise been made in broth.

Penicillin. Commercial preparations containing 100,000 units of the sodium salt of penicillin have been used for the making of stock solutions. The dry penicillin powder was dissolved in 100 ml of normal saline so as to make a concentration of 1,000 units per ml. This solution was passed through a Seitz filter and stored in the refrigerator. Studies showed that when kept in this way the penicillin solution maintained its strength for at least 3 months. The stock penicillin solutions were titrated from time to time by testing serial dilutions in broth with a stock strain of hemolytic streptococcus of known sensitivity. In all experiments broth was used for making dilutions of the stock penicillin solution.

Sulfonamide drugs. Stock solutions of the sulfonamide drugs were prepared by weighing out 20 mg of the dry powder and adding it to 100 ml of broth in which it was readily soluble. Sterilization was insured by passage through a Seitz filter.

The number of organisms in broth cultures and in broth solutions of penicillin or sulfonamide drugs was determined by making serial dilutions in broth from 10^{-1} to 10^{-7}, or in some instances, when necessary, out to as far as 10^{-12}. Exactly 0.1 ml of each dilution was added to a blood agar plate and thoroughly streaked over the surface with a wire loop. After incubation at 37 C for 48 hours, colony counts were made of those plates on which the colonies were not too crowded. From the number of colonies and the dilutions, the number of organisms per ml in the original solution or culture could be readily calculated. This method of surface streaking was found to be technically easier and just as accurate as pour plate methods. When repeated calculations are made by these methods of the number of organisms in a single culture, the results have shown a 10-fold (10^1) and sometimes even a 100-fold (10^2) variation. However, the relative error in any one experiment can be kept at a minimum by choosing blood plates on which the numbers of colonies are of about the same order. In general we have tried to choose blood plates with 200 to 1,000 colonies. When the number of organisms is being determined in cultures containing penicillin, it is important to make counts only from those dilutions in which the penicillin content has been reduced below the minimal inhibitory concentration. Excessive amounts of penicillin may interfere with the growth of the bacteria on the blood plates.

EFFECT OF VARIATIONS IN THE NUMBER OF EXPOSED ORGANISMS

Abraham *et al.* (1941), in referring to the characteristics of the antibacterial action of penicillin, stated that its action was influenced only to a minor extent by the number of bacteria to be inhibited. Hobby, Meyer, and Chaffee (1942), on the other hand, stated that with a given concentration of penicillin the rate of killing of a given strain of hemolytic streptococcus decreased as the number of organisms at zero hour increased. Fisher (1943) determined the minimal dilution of penicillin which caused inhibition, and with one strain of staphylococcus obtained the same end point with 8 billion organisms as with 17 million. Yet with a relatively resistant strain of staphylococcus he failed to get inhibition when the culture was diluted 1:1, but did get inhibition when the culture was diluted 100 times. He felt that the failure with the more concentrated culture was due to the lack of active multiplication of the organisms and pointed out that this was in accord with the views of Hobby, Meyer, and Chaffee (1942). The experience of Rantz and Kirby (1944) apparently was similar to that of Fisher. They found that very large numbers of susceptible organisms could be killed if the concentration of penicillin was large enough, but the retardation of growth of a resistant organism was more marked if the inoculum was small, and under these circumstances the cultures could be sterilized. We have investigated this problem in several different ways.

Sensitivity tests. One of the commonly used methods for determining the sensitivity of an organism involves exposing it to decreasing concentrations of penicillin for a fixed period of time such as 24 hours. The lowest concentration of penicillin (measured in units per ml) which causes inhibition represents the sensitivity of the organism. The exact way in which sensitivity tests have been performed by various investigators has varied considerably. Thus Rammelkamp and Maxon (1942), in performing tests with staphylococci, used a bacterial inoculum so that the final number of organisms was between 1,000 and 30,000 per 0.5 ml, and they reported the sensitivity of the strains tested as the minimal concentration of penicillin which produced complete killing. That is, they chose as the end point the greatest dilution of penicillin that was sterile after incubation for 18 to 24 hours. On the other hand, Dawson, Hobby, and Lipman (1944) tested the sensitivity of nonhemolytic streptococci by making a 10^{-2} or 10^{-1} dilution of the organisms in serial dilutions of penicillin. This usually produced a concentration of between 2,500,000 and 3,000,000 organisms per ml. Absence of turbidity after incubation was used as the index of inhibition. The authors pointed out that the contents of the tubes remaining clear were not necessarily sterile. Control tests were always set up with a standard stock strain of hemolytic streptococcus.

It seemed possible that the results of sensitivity tests might be influenced by the size of the inoculum and by the criteria chosen to represent "inhibition." Therefore, in order to determine this sensitivity tests were performed with several representative strains of viridans streptococci in the following way:

Experiment 1

An overnight (approximately 18 hours) broth culture was diluted serially in broth from 10^{-1} to 10^{-5} or to 10^{-6}. The number of organisms per ml in each dilution was calculated by the method already described. The 10^{-5} dilution usually was found to have between 500 and 2,000 organisms in 0.1 ml. Penicillin dilutions were prepared in broth, and 1 ml of each dilution was pipetted into each of six or seven tubes. In this way six or seven identical series of penicillin dilutions were prepared. Exactly 0.1 ml of the undiluted broth culture was added to each tube of the first series of penicillin dilutions. Then 0.1 ml of the 10^{-1} bacterial dilution was added to each tube of the second series of penicillin dilutions. This process was repeated successively with each of the bacterial dilutions so that there were obtained combinations of every bacterial dilution with every concentration of penicillin. The number of organisms per ml and the concentration of penicillin in units per ml in each tube at the start of the experiment were readily calculated since 0.1 ml of broth with a known bacterial content was added to 1 ml of a known penicillin concentration. After incubation at 37 C for between 18 and 24 hours the tubes were shaken and examined for the presence or absence of turbidity. A loop streak, or in some instances a 0.1-ml streak, on a blood agar plate was made of the contents of all tubes that remained clear in order to determine whether or not sterility had been produced. The plates were examined after 48 hours of incubation at 37 C. In this way it was possible to determine the minimal concentration of penicillin that prevented appreciable multiplication of the organisms (bacteriostasis) and the minimal concentration which completely or nearly completely killed the organisms (bactericidal effect).

In table 1A there are given the results of this experiment using the Torrice strain of viridans streptococci, which was found by preliminary tests to be relatively sensitive to the action of penicillin. The penicillin concentrations to which the organisms were exposed varied from 1.0 down to 0.01 unit per ml. It is evident that the concentration of penicillin necessary to produce sterility was very small and the same or nearly the same as that needed for bacteriostasis. Furthermore, it is also apparent that increasing the number of exposed organisms from 500 to 50,000,000 per ml caused only a slight increase in the minimal concentration of penicillin required for either a bacteriostatic or a bactericidal effect (0.02 to 0.06 units per ml).

TABLE 1A

Effect of variation in the number of exposed organisms on the penicillin sensitivity of a relatively susceptible strain of viridans streptococci

DILUTION OF OVERNIGHT CULTURE USED FOR INOCULATION	CALCULATED NUMBER OF ORGANISMS PER ML AT START OF EXPERIMENT	AFTER OVERNIGHT INCUBATION AT 37 C	
		Lowest concentration of penicillin remaining clear	Lowest concentration of penicillin showing none or very few living organisms
Undiluted	5×10^7	0.06	0.06
10^{-1}	5×10^6	0.04	0.04
10^{-2}	5×10^5	0.04	0.04
10^{-3}	5×10^4	0.02	0.04
10^{-4}	5×10^3	0.02	0.02
10^{-5}	5×10^2	0.02	0.02

In this experiment 0.1 ml of each of the indicated bacterial dilutions of the Torrice strain of viridans streptococci was added to 1 ml of the following penicillin concentrations: 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.08, 0.06, 0.04, 0.02, and 0.01 units per ml. A control tube of 1 ml of broth without penicillin was also inoculated. Since each ml of penicillin solution was further diluted by the addition of 0.1 ml of a broth culture, the concentration of penicillin was actually about 9 per cent less than the figures shown above.

TABLE 1B

Effect of variation in the number of exposed organisms on the penicillin sensitivity of a moderately resistant strain of viridans streptococci

DILUTION OF OVERNIGHT CULTURE USED FOR INOCULATION	CALCULATED NUMBER OF ORGANISMS PER ML AT START OF EXPERIMENT	AFTER OVERNIGHT INCUBATION AT 37 C	
		Lowest concentration of penicillin remaining clear	Lowest concentration of penicillin showing none or very few living organisms
Undiluted	2×10^8	0.6	Many organisms in all tubes
10^{-1}	2×10^7	0.6	Many organisms in all tubes
10^{-2}	2×10^6	0.6	Many organisms in all tubes
10^{-3}	2×10^5	0.4	Many organisms in all tubes
10^{-4}	2×10^4	0.4	0.4
10^{-5}	2×10^3	0.4	0.4
10^{-6}	2×10^4	0.4	0.4

In this experiment 0.1 ml of each of the indicated bacterial dilutions of the Abizaid strain of viridans streptococci was added to 1 ml of the following penicillin concentrations: 100, 75, 50, 25, 10, 5, 2, 1, 0.8, 0.6, 0.4, 0.2, 0.1, and 0.05 units per ml. A control tube of 1 ml of broth without penicillin was also inoculated. Since each ml of penicillin solution was further diluted by the addition of 0.1 ml of a broth culture, the concentration of penicillin was actually about 9 per cent less than the figures shown above.

When the same experiment was repeated with the Abizaid strain, a somewhat more resistant viridans streptococcus, the results were not quite the same (table 1B). In this experiment the concentrations of penicillin varied from 100 down to 0.05 units per ml. When the number of exposed organisms was 200, both a bacteriostatic and a bactericidal effect were obtained with 0.4 units of penicillin. This minimal concentration was not appreciably altered by increasing the number of organisms to 20,000, but, when the number of organisms was increased beyond this, numerous bacteria were still alive after exposure to even 100 units of penicillin. Nevertheless, the minimal concentration of penicillin required for bacteriostasis was only increased from 0.4 to 0.6 units per ml when the number of organisms was increased from as few as 200 to as many as 200,000,000 per ml. Similar tests performed with other strains of viridans streptococci have yielded comparable findings.

This experiment therefore suggests that a very susceptible strain of viridans streptococci is not only prevented from multiplying but actually killed by very low concentrations of penicillin even when the number of exposed organisms is very large. With less susceptible strains bacteriostasis can likewise be effected even when large numbers of organisms are exposed, the minimal effective concentration of penicillin, of course, being larger than that for a more susceptible strain. On the other hand, with the less susceptible strains it is difficult to produce complete sterilization even with very concentrated solutions of penicillin, especially when the number of exposed organisms is large.

Survival curves. In order to obtain more detailed information concerning the difference in behavior of a very susceptible strain and a less susceptible strain of viridans streptococci when exposed to an excess of penicillin, the following experiment was performed with the same two organisms used for the sensitivity tests just reported:

Experiment 2

In this experiment 0.4 ml of an undiluted overnight broth culture of the strain to be tested was added to 3.6 ml of a penicillin solution in broth, and then the mixture was incubated at 37 C for 48 hours. The penicillin solution was prepared so that after the addition of 0.4 ml of broth culture the final concentration was 1.0 unit per ml. Immediately after the bacteria were added to the penicillin and at various intervals thereafter for a period of 48 hours, 0.1 ml was removed and the number of organisms present was determined by the serial dilution method previously described.

Figure 1 is a graphic representation of the results of this experiment when performed with each of the strains of viridans streptococci (Torrice and Abizaid) used for the sensitivity tests. For purposes of comparison there is also shown in figure 1 the growth curve of one of the strains (Abizaid) in the absence of penicillin. The logarithm of the number of surviving organisms per ml has been plotted against the time of exposure to penicillin in hours.

From the curve of the less susceptible strain (Abizaid) it is evident that the number of surviving organisms gradually became less, and at an approximately constant rate. At the end of 48 hours there were still a good many viable bacteria present. The more susceptible strain (Torrice) decreased much more rapidly, so that within 12 hours the original concentration of 10,000,000 bacteria per ml was reduced to 70 viable organisms per ml. Up to the twelfth hour the rate of decrease was more or less constant, but after the twelfth hour the few remaining organisms disappeared more slowly. However, by 24 hours there were only 10 viable organisms per ml, and by 48 hours the penicillin solution was completely sterile.

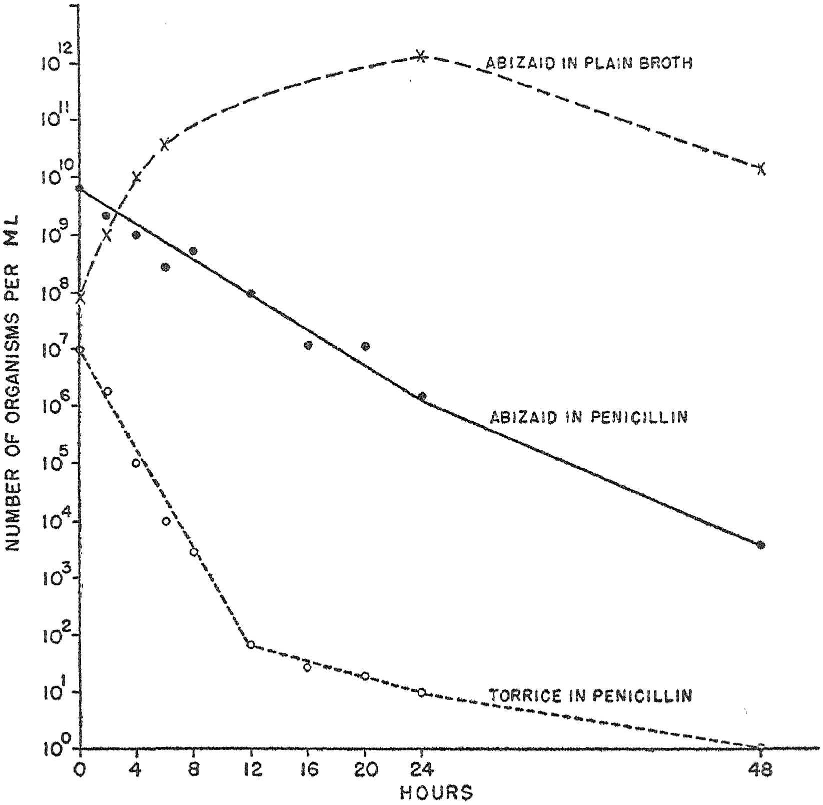

FIG. 1. SURVIVAL CURVES OF A VERY SUSCEPTIBLE AND A MORE RESISTANT STRAIN OF VIRIDANS STREPTOCOCCI IN THE PRESENCE OF 2 UNITS OF PENICILLIN

The number of organisms per ml have been charted logarithmically. The penicillin sensitivity of the Abizaid strain was about 0.35 units, and that of the Torrice strain about 0.035 units. Both strains were exposed to about 2 units of penicillin in broth. For comparison there is also shown the growth curve of the Abizaid strain in broth without penicillin.

This experiment, therefore, suggests that the less susceptible strain of viridans streptococci (Abizaid) differed from the more susceptible strain (Torrice), not only in that the minimal concentration of penicillin necessary for bacteriostasis was greater, but also in that, even in the presence of an excess of penicillin, the rate at which the organisms were killed was slower. We have also determined the survival curves of other strains of viridans streptococci and have found several moderately resistant ones to behave similarly to the Abizaid strain. However, of four susceptible strains whose bacteriostatic sensitivities fell within the range of 0.04 to 0.08 units, only one showed a rate of decrease which was at all comparable to that of the Torrice strain. From our data, therefore, we are not yet able to determine whether the rate of decrease of organisms in the presence of an excess of penicillin is correlated in any way with the minimal concentration of penicillin necessary to prevent multiplication (bacteriostatic sensitivity).

Experiment 2 also clarifies the results of the previously described sensitivity tests (experiment 1). The rate with which the Abizaid strain is killed even in the presence of an excess of penicillin is too slow to produce sterilization at the end of 24 hours unless very few organisms are present at the start. On the other hand, the rapid rate with which the Torrice strain is killed by an excess of penicillin makes understandable the fact that a culture containing even large numbers of organisms can be completely or nearly completely sterilized within 24 hours.

Of interest is the change in the slope of the curve for the Torrice strain after all but 70 organisms per ml had been killed (figure 1). This type of effect has previously been reported by Hobby, Meyer, and Chaffee (1942), who concluded that the number of survivors decreased by geometric units as the time increased by arithmetic units. Thus they found that the log of the number of survivors when plotted against time followed a straight line until 99 per cent of the organisms were killed. It should be noted that even with the more resistant Abizaid strain the number of viable organisms had been reduced from 7,000,000,000 at zero hour to 1,500,000 at the end of 24 hours. Although this represents a reduction of well over 99 per cent and is therefore confirmatory of the observations reported by these investigators, nevertheless the absolute number of survivors is large.

The small number of organisms of even a susceptible strain that fail to get killed readily are referred to by Bigger (1944*b*) as "persisters." However, these remaining organisms cannot multiply so long as they are exposed to the action of penicillin, and eventually they may die of their own accord. On the other hand, if the penicillin is destroyed or for some other reason is reduced below the minimal concentration necessary for inhibition, even a few remaining viable organisms may rapidly multiply again. This is of some practical importance if the *in vivo* action of penicillin is at all comparable to that observed *in vitro*.

Effect of variations in concentration of the organisms on the rate of decrease. From figure 1 it is evident that the less susceptible Abizaid strain of viridans streptococci, even in the presence of an excess amount of penicillin, was killed relatively slowly. In this particular experiment a fairly large inoculum was used so that at zero hour there were 7×10^9 organisms per ml. In order to determine whether or not the number of organisms influenced the rate of killing the following type of experiment was performed:

Experiment 3

A solution of penicillin in broth was prepared so that after 0.2 ml of an undiluted overnight broth culture of the Abizaid strain had been added to 1.8 ml of the solution, the final concentration of penicillin was 2 units per ml. By making appropriate serial dilutions of this with broth containing 2 units of penicillin per ml, we obtained a series of ten tubes, each of which contained a total volume of 1 ml, 2 units of penicillin, and in sequence the following dilutions of the original overnight broth culture: 10^{-1}, 0.5×10^{-1}, 10^{-2}, 0.5×10^{-2}, 10^{-3}, 0.5×10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. The number of organisms present in each dilution immediately after preparation was determined by the method previously described. The tubes were then placed in the incubator at 37 C, and after 24 hours the number of viable organisms per ml in each tube was again determined. As a control a duplicate set of tubes was prepared with broth not containing penicillin and incubated simultaneously.

In all of the control tubes that did not contain penicillin, the number of viable organisms at the end of 24 hours had increased to approximately the same final concentration, the range of variation being between 1.5×10^{11} and 5×10^{12} organisms per ml.

The results of the tests with the penicillin broth cultures are shown by the graph on the left-hand side of figure 2, where the calculated number of organisms per ml at zero hour and the number after 24 hours' incubation in the presence of 2 units of penicillin are recorded logarithmically. The corresponding points for each tube have been connected by straight lines. The slope of the line for each tube represents the rate of decrease of viable organisms. It should be borne in mind that these rates are necessarily only approximate because of the relatively large chance for error in calculating the number of organisms per ml by the dilution method.

For purposes of confirmation, the same type of experiment was repeated with the Miller strain of viridans streptococci, which was found by previous tests to have a sensitivity of 0.4 units per ml, a value similar to that for the Abizaid strain. The tests with the Miller strain were performed with 1 unit of penicillin per ml and seven different dilutions of the overnight broth culture. The number of organisms per ml at zero hour and again after 24 hours' incubation at 37 C are also shown graphically in figure 2.

It would appear from figure 2 that the number of organisms at zero hour, at least within the range of the bacterial concentrations tested, does not influence appreciably the rate of bactericidal activity of an excess of penicillin. The slopes of the lowest two curves for both the Abizaid strain and the Miller strain suggest that the rate of killing may diminish slightly when the number of exposed organisms becomes relatively small. These conclusions have been borne out by our experience on many other occasions with these same two strains (Abizaid and Miller) and by tests of several other relatively resistant strains.

RELATION OF THE BACTERICIDAL ACTIVITY OF PENICILLIN TO ITS CONCENTRATION

Abraham and his associates (1941), in referring to the plate method for assaying penicillin, showed that the diameter of the zone of inhibition did not increase appreciably after the concentration of penicillin was increased above a certain amount. Hobby, Meyer, and Chaffee (1942) likewise demonstrated in experiments with hemolytic streptococci that with a given number of organisms the rate of killing increased within limits as the concentration of penicillin was increased, but there was a point beyond which increases in concentration of penicillin no longer increased the rate of killing. On the other hand, Fisher (1943) stated that the speed and degree of action of penicillin depended on its strength.

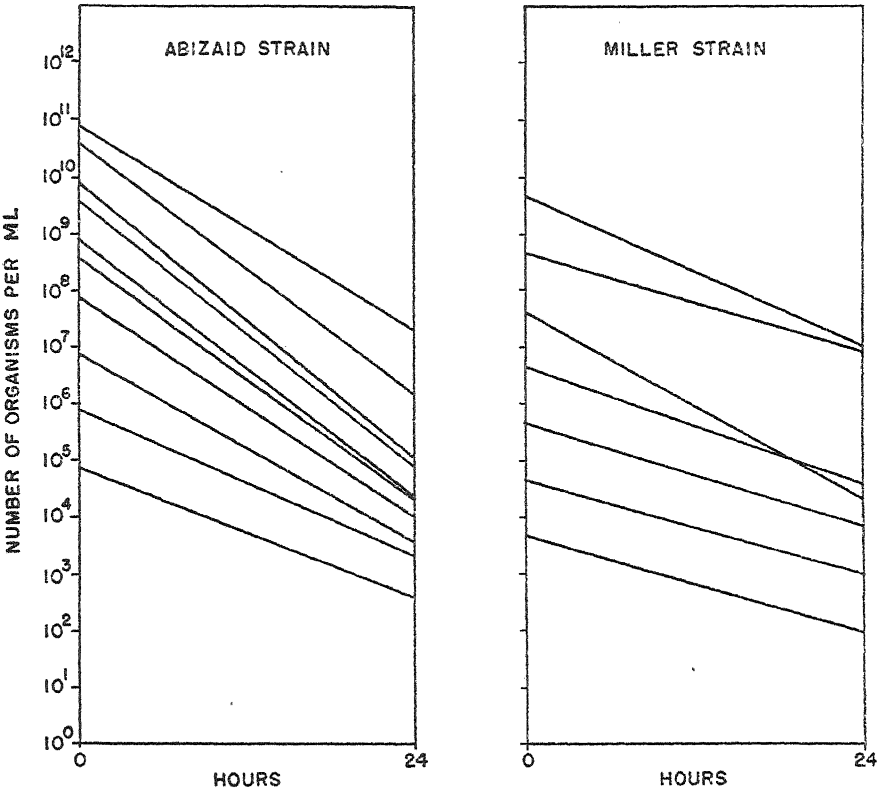

FIG. 2. EFFECT OF VARIATIONS IN THE NUMBER OF ORGANISMS ON THE BACTERICIDAL ACTION OF PENICILLIN

The number of organisms are charted logarithmically. Determinations were made at zero hour and again after incubation for 24 hours. The zero and 24-hour points have been connected by straight lines. The Abizaid strain was exposed to 2 units of penicillin per ml and the Miller strain was exposed to 1 unit.

Miller, Green, and Kitchen (1945) also found that inhibition was directly related to the penicillin concentration. Rantz and Kirby (1944) in the body of their paper stated that above a certain concentration the activity of penicillin was not enhanced by increasing amounts of the drug. Yet in the summary of this same paper they said that "there is a close correlation between the concentration of penicillin and its activity."

From the sensitivity tests of experiment 1 (table 1A) and from the survival curve shown in figure 1, it is apparent that even a very heavy inoculum of a very sensitive organism, such as the Torrice strain, can be almost completely destroyed within a relatively short period. When such bacteria are concerned, it is obvious that all one needs to be sure of is that the penicillin be maintained above the minimal concentration necessary for inhibition. On the other hand, when one is dealing with organisms such as the Abizaid and Miller strains, which are not killed so rapidly, a question which might be of some practical importance is whether the rate of destruction or the number of organisms destroyed can be enhanced by increasing the concentration of penicillin above the minimal concentration needed to prevent growth. Since an unequivocal answer could not be obtained from a review of the medical literature, the following experiment was performed:

TABLE 2

Effect of variation in the concentration of penicillin on its bactericidal activity

PENICILLIN UNITS PER ML	NUMBER OF ORGANISMS PER ML	
	At zero hour	At 24 hours
7.3	1×10^9	2.3×10^5
3.6	1×10^9	1.9×10^5
1.8	1×10^9	1×10^5
0.9	1×10^9	1.2×10^5
0.46	1×10^9	1.2×10^5
0.23	1×10^9	2×10^9
0	1×10^9	1.5×10^{12}

Calculations of the concentration of penicillin and the number of organisms at zero hour take into consideration the fact that 0.2-ml amounts of a 10^{-1} dilution of a broth culture were added to 2-ml volumes of penicillin in broth. The Abizaid strain which was used in this experiment had previously been determined to have a sensitivity of about 0.35 units.

Experiment 4

In a series of tubes 2-ml volumes of broth were prepared so that they contained the following concentrations of penicillin: 8, 4, 2, 1, 0.5, 0.25, and 0 units per ml. Each tube was inoculated with 0.2 ml of a 10^{-1} dilution of an overnight broth culture of the Abizaid strain of viridans streptococci. It is apparent that the concentration of penicillin in each tube was reduced by approximately 9 per cent by the addition of the broth culture, so that the final penicillin concentrations were 7.3, 3.6, 1.8, 0.9, 0.46, 0.23, and 0 units per ml. The number of organisms in the inoculum, and hence the number of organisms per ml in each tube at zero hour, were calculated by the serial dilution method. The tubes were placed in an incubator at 37 C, and the number of viable organisms per ml in each tube was determined at the end of 24 hours. Previous experiments had shown the Abizaid strain to have a penicillin sensitivity of about 0.35 units.

The results of this experiment are shown in table 2. With 0.23 units of penicillin, a concentration slightly under the "sensitivity" of the Abizaid strain, there seems to be some bacteriostasis but no appreciable killing. On the other hand, with concentrations of 0.46 units of penicillin and higher there is a definite reduction in the number of viable organisms during the 24-hour period. It is evident that the degree of reduction is about the same in all of the first five tubes. Hence it appears that above a critical concentration even a 16-fold increase in the concentration of penicillin (0.46 to 7.3 units) caused no increase in the number of organisms killed. Many similar experiments with this same strain of viridans streptococci and with other strains have yielded similar results.

EFFECT OF SULFONAMIDE DRUGS ON THE ACTION OF PENICILLIN

Ungar (1943) reported that the inhibitory effect of penicillin in the presence of small quantities of sulfapyridine, which per se were insufficient to show any effect, was at least double. The *in vitro* tests were made with staphylococci and streptococci, and the activity of the penicillin was determined by observing the highest dilution which caused inhibition of growth. Ungar also confirmed these conclusions by *in vivo* experiments with mice. Rammelkamp and Keefer (1943) observed that the addition of small amounts of penicillin, which in itself displayed no killing effect against staphylococci, seemed to enhance the effectiveness of sulfathiazole in whole defibrinated blood. An apparent synergism of sulfonamide drugs and penicillin was likewise found by T'Ung (1944) in experiments with sulfathiazole, penicillin, and *Brucella* organisms, and by Bigger (1944*a*) in experiments with staphylococci, penicillin, and various sulfonamide drugs. Bigger concluded that sulfathiazole was more effective as a synergistic agent against the staphylococcus than sulfanilamide and sulfapyridine, and he found that the degree in which the penicillin activity was enhanced by sulfathiazole varied with the strain. *In vivo* experiments on mice by Soo-Hoo and Schnitzer (1944) also suggested that sulfonamide drugs increased the effectiveness of penicillin as an antistreptococcal agent.

In contrast to these reports, Hobby and Dawson (1944*a*, 1944*b*, 1945) found no evidence of a synergistic action between sulfadiazine or sulfapyridine and penicillin. They pointed out that the activity of penicillin is decreased by conditions which slow the rate of multiplication of the organism. Furthermore, they observed that sulfonamides decreased the rate of multiplication and hence actually tended to decrease the rate at which penicillin acted. Kirby (1944) also conducted *in vitro* experiments on the bacteriostatic action of sulfonamide-penicillin mixtures. He concluded that the effect of penicillin plus sulfadiazine was equal to the effect of penicillin alone plus the effect of sulfadiazine alone. That is, there was no actual synergism or potentiation, but the effects were additive.

Because of these apparently contradictory findings regarding the effect of sulfonamides on the action of penicillin, experiments of the following type were performed:

Experiment 5

Serial dilutions of penicillin in 2-ml volumes were prepared in plain broth and in broth containing sulfamerazine.[1] The concentrations of penicillin were 1.0, 0.5, 0.25, 0.125, 0.06, and 0 units per ml. The final concentration of the sulfamerazine was 10 mg per 100 ml.

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[1] The sulfamerazine was supplied through the courtesy of Dr. J. W. Crosson of the Sharp and Dohme Company.

Each of the 12 tubes was then inoculated with 0.2 ml of a 10^{-3} dilution of an overnight culture of a strain of viridans streptococcus, designated A2, the sensitivity of which had previously been found to be approximately 0.25 units. It is apparent that by adding 0.2 ml of broth culture to 2 ml of penicillin solution the concentration of penicillin in each tube was reduced by approximately 9 per cent. Thus the actual concentrations of penicillin after addition of the bacteria were 0.91, 0.45, 0.23, 0.11, 0.05, and 0 units of penicillin per ml. The actual sulfamerazine concentration was also reduced to approximately 9.1 mg per 100 ml. The number of organisms per ml in each tube was determined by the method previously described immediately after inoculation and again after incubation of the tubes for 24 hours at 37 C.

The results of this experiment are shown in table 3. It is evident that, in the absence of sulfamerazine, concentrations of penicillin below 0.23 units per ml had very little inhibitory effect on the growth of the bacteria. The organisms multiplied readily, although there is a suggestion that the rate of multiplication may have been slightly slower than in the control tube containing no penicillin. It is also evident that a fourfold increase in the concentration of penicillin from 0.23 units to 0.91 units per ml did not accelerate the rate of destruction of the bacteria. The latter finding is similar to that demonstrated by experiment 4, the results of which are shown in table 2.

TABLE 3

Bactericidal effect of various concentrations of penicillin in the presence and in the absence of sulfamerazine

PENICILLIN UNITS PER ML	SULFAMERAZINE MG PER 100 ML	NUMBER OF ORGANISMS PER ML	
		At zero hour	At 24 hours
0.91		1.2×10^6	7×10^2
0.45	0	1.1×10^6	4×10^2
0.23	0	1.0×10^6	1×10^2
0.11	0	0.8×10^6	3×10^9
0.05	0	0.8×10^6	2×10^9
0	0	0.8×10^6	1×10^{10}
0.91	9.1	0.8×10^6	5×10^2
0.45	9.1	0.8×10^6	2×10^2
0.23	9.1	0.7×10^6	1.5×10^2
0.11	9.1	0.8×10^6	2×10^3
0.05	9.1	0.8×10^6	$2 \times 10_4$
0	9.1	0.8×10^6	2×10^9

Calculations of the concentration of penicillin and the concentration of sulfamerazine take into consideration the fact that 0.2-ml amounts of a 10^{-3} dilution of a broth culture were added to 2-ml volumes of penicillin in broth or to penicillin and sulfamerazine in broth. The strain used in this experiment had a sensitivity of about 0.25 units.

With the minimal inhibitory penicillin concentration of 0.23 units and in concentrations above this, the presence of sulfamerazine did not appreciably increase or decrease the bactericidal activity of the penicillin, the decrease in the number of organisms being approximately the same in those tubes containing sulfamerazine as in those without the drug. However, in the presence of sulfamerazine a concentration of 0.11 units of penicillin, which in the absence of sulfamerazine had been ineffective, caused almost as great destruction of bacteria as 0.23 units. Even 0.05 units had some bactericidal activity in the presence of sulfamerazine. It is evident that the sulfamerazine in the absence of penicillin had extremely slight if any inhibitory effect on the growth of this strain of viridans streptococci.

Thus it would appear that under the experimental conditions the presence of sulfamerazine caused a lowering of the minimal concentration of penicillin necessary to produce both bacteriostasis and destruction of the organisms. Other similar experiments with the same strain and with other strains have yielded practically identical results. However, we have encountered some strains of viridans streptococci for which this apparent synergism could not be demonstrated.

In table 4 we have compared the minimal bacteriostatic concentrations of penicillin for different strains of viridans streptococci in the presence and in the absence of sulfonamides. It is evident that when sulfonamides enhanced the action of penicillin the minimal bacteriostatic concentration was reduced anywhere from about 25 to 75 per cent, depending on the strain.

TABLE 4

Penicillin sensitivity of different strains of viridans streptococci when tested with penicillin alone and with penicillin plus sulfonamides

STRAIN	MINIMAL CONCENTRATION OF PENICILLIN (IN UNITS PER ML) NECESSARY TO CAUSE A BACTERIOSTATIC EFFECT	
	In the absence of sulfonamides	In the presence of sulfonamides
Miller	0.40	0.20
Abizaid	0.35	0.15
A2	0.23	0.05
W2	0.10	0.10
Torrice	0.035	0.025

We have compared this synergistic activity of sulfathiazole, sulfadiazine, and sulfamerazine and have found no significant difference. We have also performed tests with various concentrations of the sulfonamide drugs and have found a concentration of 5 mg per 100 ml to be nearly if not actually as effective as 10 mg per 100 ml, but concentration below 5 mg per 100 ml became decreasingly effective.

DISCUSSION AND CONCLUSIONS

The data presented in this report suggest that when the viridans streptococci are incubated in the presence of increasing concentrations of penicillin in broth a critical concentration is reached at which the bacteria fail to multiply. Further increases in the concentration of penicillin cause a diminution of the number of bacteria. These findings are consistent with the fact, now well established by many investigators, that penicillin produces a bactericidal as well as a bacteriostatic effect. The critical concentration at which multiplication is prevented varies for different strains, and for any particular strain it is a measure of the organism's penicillin sensitivity.

Increases in the concentration of penicillin beyond that necessary for bacteriostasis produce increases in the rate at which the bacteria are killed until another critical concentration is reached beyond which further increases fail to manifest any greater bactericidal activity. These observations are confirmatory of those reported by Hobby, Meyer, and Chaffee (1942). Thus we have found that when a relatively resistant strain of streptococcus is involved, the rate at which the organisms are killed cannot be accelerated by exposing the bacteria to even very high concentrations of penicillin.

On the basis of our own observations as well as from data found in the medical literature, it seems likely that the difference between the minimal concentration necessary for bacteriostasis and the minimal concentration necessary for a maximum bactericidal effect is relatively small for most bacteria. However, our experience with at least one strain of viridans streptococci suggests that the difference may occasionally be relatively large, although we have made no attempt as yet to study this aspect of the problem in any great detail.

Our observations on strains of viridans streptococci incubated in the presence of bactericidal concentrations of penicillin confirm the statement of Hobby, Meyer, and Chaffee (1942) that the log of the number of survivors plotted against time follows a straight line until at least 99 per cent of the organisms are killed. The slope of this line that represents the rate of decrease varies for different strains. For all of the relatively resistant strains which we have studied (minimal bacteriostatic concentration of penicillin = 0.1 unit per ml or more), the slope of the logarithmic survival curves have been relatively shallow. Thus, one relatively resistant strain was reduced in a period of 24 hours from 1,000,000 to approximately 2,000 organisms per ml, and another relatively resistant strain was reduced from 4,000,000 to about 20,000 organisms per ml (figure 2). Although the number of survivors in each instance represents less than 1 per cent of the original inoculum, nevertheless the absolute number is still fairly large.

Some very susceptible strains (minimal bacteriostatic concentration of penicillin 0.04 units or less) have been found to be killed very rapidly in the presence of an excess of penicillin, and their logarithmic survival curves are steep. Thus in one particular instance an inoculum of 3,000,000 organisms per ml was reduced to 20 viable organisms in the course of 24 hours (Torrice strain in figure 1). However, several other strains whose bacteriostatic sensitivities ranged between 0.04 and 0.08 units of penicillin were found to be killed more slowly and at a rate which was comparable to that of the more resistant strains.

We have not yet had the opportunity to study a sufficient number of strains of viridans streptococci in order to determine whether or not there is any relation between the maximum rate of killing (slope of the logarithmic survival curve) and the minimal concentration of penicillin necessary for bacteriostasis (sensitivity). Whatever might be the outcome of such observations, it seems to us that the maximum rate at which bacteria can be killed by penicillin is a quality which may be of some importance, and which may have some bearing on the response of patients with subacute bacterial endocarditis to treatment with penicillin. It seems possible that some of the failures of penicillin therapy might be related to the fact that the causative organisms, though prevented from multiplying, were killed very slowly and hence survived for long periods of time even in the presence of concentrations of penicillin above the sensitivity level. This possiblity, though only conjectural, deserves further investigation.

Our observations, in confirmation of the statement made by Abraham *et al.* (1941), suggest that the number of organisms do not alter appreciably the action of penicillin. When a very susceptible strain of viridans streptococci is involved, all of the organisms, or all but a very few "persisters," are killed within 24 hours, even when the original inoculum is large. When the strain under observation is killed more slowly, as is true of the more resistant strains which we have observed, the rate of decrease in the number of viable organisms in the presence of an effective concentration of penicillin is not appreciably altered by wide variations in the size of the inoculum. The number of viable organisms present after incubation will depend upon the size of the original inoculum. If the inoculum of such a strain is large, many viable organisms will still be present at the end of 24 hours. However, if the inoculum is very small, the rate of decrease may be sufficient to bring about complete sterility at the end of 24 hours, though usually the presence of at least a few viable organisms can still be detected.

These studies throw some light on the use and interpretation of sensitivity tests. If to a series of tubes containing different concentrations of penicillin there is added a bacterial inoculum of such size that the tubes still appear clear, then after incubation for 24 hours the presence or absence of turbidity can be used as the criteria for determining the end point. Those tubes that are turbid after shaking obviously contain concentrations of penicillin in which the organisms readily multiplied. The lowest concentration of penicillin remaining clear is the minimal concentration which prevents appreciable multiplication and therefore represents the bacteriostatic sensitivity of the organism. This is essentially the method used by Dawson, Hobby, and Lipman (1944) for determining the sensitivity of various strains of nonhemolytic streptococci.

The presence or absence of viable organisms in the clear tubes depends on the size of the original inoculum and the rate at which the organisms are killed. If the strain is one which is killed slowly and the original inoculum is relatively large, such as might be obtained with a 10^{-1} or a 10^{-2} dilution of the overnight culture, then it is likely that many viable organisms will still be present in all of the clear tubes. If the original inoculum of the same strain is small, such as might be obtained with a 10^{-5} or 10^{-6} dilution of the overnight culture, then the tubes remaining clear after incubation may be sterile or contain very few viable organisms. If the strain being tested is one which is killed rapidly by effective concentration of penicillin, then the tubes remaining clear after 24 hours of incubation will be either sterile or contain only a few viable organisms, and this will be so irrespective of the size of the original inoculum. Therefore it is apparent that if one wishes to obtain satisfactory information regarding both the rate at which a particular strain can be killed and the minimal concentration of penicillin producing the maximum rate of killing, it is necessary to determine the number of organisms in the original inoculum and also in the tubes remaining clear after incubation.

Our *in vitro* studies with sulfonamides are confirmatory of the original observations of Ungar (1943). We have found that with many strains of viridans streptococci the minimal concentration of penicillin necessary to produce either a bacteriostatic or a bactericidal effect can be reduced 25 to 75 per cent by the presence of sulfonamide drugs. In other words, a subeffective concentration of penicillin in which the bacteria readily multiply may, in the presence of sulfonamides, cause just as rapid killing of the organisms as a more concentrated preparation of penicillin. However, if penicillin is present in a concentration well above that of the sensitivity of the strain of viridans streptococci being tested, sulfonamides will not alter appreciably the rate at which the organisms are killed.

SUMMARY

From studies of the *in vitro* action of penicillin on various strains of viridans streptococci the following observations have been made:

1. Concentrations of penicillin above the minimal concentration necessary for bacteriostasis have a bactericidal action. For any given strain there is a critical concentration of penicillin beyond which further increases in concentration have no increased bactericidal activity.
2. Strains of viridans streptococci differ not only in regard to the minimal concentration of penicillin necessary for bacteriostasis but also in regard to the rate at which they are killed in the presence of an excess of penicillin. There is need for further studies concerning the possible correlation of these two variables.
3. Within wide limits, variations in the number of exposed organisms do not appreciably alter the rate of bactericidal action of penicillin.
4. Sulfonamide drugs seem to have the following effect on the *in vitro* action of penicillin: (a) The minimal effective concentration of penicillin is reduced for many strains of viridans streptococci. (b) In the presence of penicillin in amounts above the minimal effective concentration, sulfonamides do not appreciably alter the bactericidal action of the penicillin.

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STRAIN VARIATION AS A FACTOR IN THE SPORULATING PROPERTIES OF THE SO-CALLED BACILLUS GLOBIGII[1]

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For a period of more than fifty years interest has been evidenced in the environmental factors responsible for endospore formation in bacteria. Lehmann (1888) attributed sporulation to the accumulation of metabolites in the medium, but Buchner (1890) held that exhaustion of the nutrients was the cause of spore formation. Subsequent reports in the literature were adequately reviewed by Brunstetter and Magoon (1932). Additional investigations include those of Williams (1930-31), Cook (1931), Bayne-Jones and Petrilli (1933), Fabian and Bryan (1933), Roberts (1934), Roberts and Baldwin (1942), Hayward (1943), and Knaysi (1945).

Our original purpose was to investigate some of the environmental factors which influence the sporulation of "*Bacillus globigii.*" In the course of these investigations we encountered serious difficulty in repeating, or even in duplicating, experimental data. It was noticed that considerable colonial variation took place when this organism was cultured in liquid media. The following experiments were designed to test the premise that various strains, as determined by colonial appearance, possess varying ability to sporulate under similar environmental conditions.

PROCEDURES

The organism used was a strain of *B. globigii*, originally obtained from the University of Wisconisn. This bacillus produces a deep orange pigment and grows well on nutrient agar. However, it was found that pigmentation was enhanced when *B. globigii* was grown on a medium made of corn steep liquor (a by-product of the starch-refining industry); hence this medium was used.

The corn steep liquor was clarified by raising the pH to 8.0 with 50 per cent NaOH, heating for 30 minutes at 70 to 80 C, and filtering through a Buchner funnel precoated with filter-cel no. 540 (a diatomaceous earth filter aid made by the Johns-Manville Company). The filtrate was adjusted to pH 7.2 with H_2SO_4, and the total solids were determined by drying triplicate aliquots in tared dishes at 110 C to constant weight. Sufficient filtrate was diluted with distilled water to make a solution containing 2.5 per cent of total solids. Additional nutrients such as peptones, glucose, and starch were added directly to this solution in the desired concentration, and the medium was sterilized by autoclaving at 125 C for 30 minutes.

Preliminary work was done with liquid media in 500-ml gas-washing bottles equipped with sintered glass spargers through which air was passed by means of a vacuum. This technique, however, was extremely difficult to control and recourse was had to agitation as a means of aeration. For this purpose a reciprocal shaking machine was constructed capable of holding six trays, each tray containing thirty-two 125-ml Erlenmeyer flasks. The machine was cam-driven, powered with a $\frac{3}{4}$-hp motor, and delivered 60 complete strokes per minute with a stroke length of 6 inches. The amount of aeration could be controlled approximately by varying the amount of liquid in the flasks. We found that adequate aeration was obtained by using 20 ml of liquid in each flask. The machine was placed in a constant temperature room maintained at 33 ± 1 C.

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[1] Studies conducted at Camp Detrick, Frederick, Maryland, from May to July, 1945.

[2] First Lt., First Lt., and Capt., respectively.

The use of agitation as a means of aerating liquid cultures proved of great value. Many more experiments could be conducted than was possible with aeration trains, and close agreement between replicate experiments was characteristic.

TABLE 1

Comparison of sporulation of five strains of Bacillus globigii on six different liquid substrates

MEDIA	PERCENTAGE OF SPORULATION OF INDICATED STRAINS, 21, 28, AND 45 HOURS AFTER INOCULATION														
	Strain F_1			Strain F_2			Strain R_1			Strain R_2			Strain S		
	21	28	45	21	28	45	21	28	45	21	28	45	21	28	45
2.5% corn steep liquor	5	20	95	5	20	90	1	5	50	1	2	40	0	2	40
2.0% corn steep liquor 0.5% peptone	40	70	95	25	40	90	1	5	40	1	2	60	0	2	20
2.0% corn steep liquor 0.5% peptone 0.5% glucose	2	5	95	0	2	85	0	2	60	0	2	70	0	2	50
2.0% corn steep liquor 0.5% peptone 0.5% starch	10	15	95	15	15	95	0	2	20	0	2	20	0	2	10
2.0% corn steep liquor 0.5% glucose	2	2	95	0	5	95	0	2	30	0	2	60	0	2	40
2.0% corn steep liquor 0.5% starch	20	30	95	5	10	90	0	2	20	0	2	10	0	2	30

The percentage of spores was estimated microscopically by the examination of films stained by the Gram technique as well as by examination of wet preparations. This technique had been used previously by one of us (Hayward), and its limitations, which are adequately discussed by Knaysi (1945), are known. Final accuracy is not claimed for the figures in table 1, but the trends shown are considered fully significant.

EXPERIMENTAL

Colonial variants were obtained by smearing a number of plates of a medium composed of 2.5 per cent CSL (corn steep liquor) and 2 per cent agar with dilutions of an old liquid culture of *B. globigii.* The plates were examined under a dissecting microscope with reflected light at 6× magnification after 20 hours' incubation. It was important to examine the plates while the colonies were relatively young and when the plates were not crowded, otherwise all the colonies had a similar appearance and the variants were difficult to distinguish. Nonpigmented and mucoid colonies were disregarded because it was desired to retain the typical pigmentation of the species. Five different colonial types were isolated and characterized as follows:

(1) S type colony. A smooth, circular, glistening colony with entire edge, umbonate elevation, and with buttery consistency.
(2) R_1 type colony. A rough colony, circular in shape, glistening, with undulate margin, umbonate elevation, and buttery consistency.
(3) R_2 type colony. Differentiated from the R_1 type by deeper serrations and a more coarsely granular appearance.
(4) F_1 type colony. A flat, very coarsely granular colony with curled edge, generally circular, and lacking the glistening qualities of the S and R types.
(5) F_2 type colony. Differentiated from the F_1 type by a comparative lack of pigment and more tenacious adherence to the agar medium.

In the parent culture the R_1 colonies predominated, whereas the F types were encountered less than once in each thousand colonies observed. Although no special effort was made to determine the variation pattern, it was noticed that R types would vary to S. The reverse was not encountered, and the S as well as the F type colonies were relatively stable.

The sporulating qualities of the five strains were determined by inoculating flasks of 2.5 per cent CSL medium supplemented with peptone or carbohydrate, or both, with approximately 1.0×10^5 spores of the respective strains. The flasks were agitated on the shaking machine described above, and examination of the contents was made at periodic intervals. The results of a typical experiment are shown in table 1. It will be seen that the F_1 and F_2 strains sporulated much faster and more completely than the R and S strains on the six media combinations tested. The composition of the substrate exerted some influence, but our data are incomplete. It is hoped that a more complete report on this phase of the problem can be made in the future.

The objection might be raised that the percentage figures quoted do not represent a true picture because of the lysis of vegetative cells in the F strains. This objection is not valid however, because the total spore crop of the F_1 strain in these media was approximately 1.0×10^9 spores per ml, whereas the total count for the R_1 strain rarely exceeded 0.5×10^9 spores per ml. All spore counts were made by averaging the counts of quadruplicate plates having at least 50 colonies per plate.

DISCUSSION

It was determined that various strains of *B. globigii* possessed different degrees of ability to sporulate under a given set of environmental conditions. This fact led to difficulty in duplicating experiments and thus to doubtful interpretation of data. By the selection of a relatively stable strain (F type) having good sporulating ability, together with the use of controlled agitation as a means of aeration, it was possible to duplicate results, so that the standard deviation of replicate experiments was of the same order as the standard deviation of the poured plate and direct microscopic counting procedures used.

SUMMARY

It is shown that variant strains of the so-called *Bacillus globigii* possess varying degrees of ability to sporulate under similar environmental conditions.

Positive correlation between colonial morphology and sporulating ability is shown for *B. globigii.*

The importance of using a pure and stable strain of an organism for studies on physiological factors affecting endospore formation is indicated.

Favorable evidence is presented for the use of agitation as an aeration procedure, and a brief description is given of a machine designed for this purpose.

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SAMPLING DEVICES FOR AIR-BORNE BACTERIA

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Any measures which effect a reduction in respiratory infections are worthy of serious consideration. There is increasing clinical evidence that some of these infections may be reduced by the proper application of germicidal ultraviolet energy (Wheeler *et al.*, 1945; Wells, 1945; Robertson *et al.*, 1943; Luckiesh and Holladay, 1942). Quartz mercury arcs have been used in the laboratory for many years for sterilizing and disinfecting purposes, but they are not suitable for general application in the field of air disinfection. The newest source of germicidal energy, the so-called germicidal lamp, consisting of a low-pressure mercury arc in a special ultraviolet-transmitting glass tube, has a germicidal efficiency 5 to 10 times that of the most potent quartz mercury arc. These germicidal lamps are relatively powerful sources of germicidal ultraviolet energy, approximately 25 per cent of the input wattage being radiated in the spectral region of $\lambda 2537$, which is approximately of maximal germicidal effectiveness.

The efficacy of a system of air disinfection, such as the use of germicidal lamps, can be appraised by clinical or physical methods. Both have important limitations. The clinical method requires adequate control tests, and, notwithstanding the best planning and experimental technique, there are uncontrollable factors. Clinical evidence of the value of ultraviolet energy in this field is already available as the result of many researches, however, and furnishes convincing proof of its value when used in adequate intensities.

Devices of varying degrees of sensitivity for the measurement of germicidal energy ($\lambda 2537$) have been developed in this laboratory (Taylor, 1944, 1945). They make possible an engineering foundation for the use of germicidal lamps. They also make it possible to check on the depreciation of lamps by reason of age, dust, etc. One of these devices can be used to determine whether the intensities at bed level in a hospital are in conformity with values established by the Council on Physical Therapy of the American Medical Association.

Physical appraisal of the presence of air-borne microorganisms, and of the efficacy of their removal by any system of disinfection, requires the application of satisfactory methods of air sampling. Considered in the light of the results achieved, air samplers now in use may be divided into two general classifications; viz., those which collect the organisms without breaking up possible clumps of bacteria, and those which break up such clumps so that each organism has a chance to grow a colony on the culture medium. Some samplers of the first type are the Wells (1933) air centrifuge, the funnel-type sampler of Hollaender and Dalla Valle (1939), Bourdillon's (1941) slit sampler, the sieve device of duBuy and Crisp (1944), and the funnel device as modified by Berry (1941) to add the effect of an electrostatic field. Two samplers which probably break up clumps of bacteria, at least partially, are the bead bubbler of Wheeler, Foley, and Jones (1941) and the atomizer-bubbler device of Moulton, Puck, and Lemon (1943). The devices which break up the clumps collect the bacteria in water, from which they must be plated out. The other devices collect the microorganisms directly on the culture medium, which can then be incubated for colony formation. Most of these devices have been extensively studied by the Industrial Hygiene Research Laboratory, National Institute of Health (duBuy, Hollaender, and Lackey, 1945).

In order to be most useful in sampling air-borne bacteria, it would appear that an air sampler should have certain characteristics, among which the following are probably the most important:

(a) *High efficiency*, collecting a large percentage of all microorganisms entering it, *regardless of their origins*.
(b) *Simplicity of operation*, preferably being a complete apparatus requiring no auxiliary equipment such as vacuum pump, manometer, etc.
(c) Use of *standard petri dishes* with solid culture media, to avoid the necessity of "plating out" from liquids.
(d) *Freedom from bacterial contamination* which would require sterilization between samplings.
(e) A *constant* and known *air rate*, so that a definite amount of air is sampled in a given time.
(f) A high degree of *portability*, so that samplings can be made conveniently and simply wherever desired.

During the course of six years of fundamental research the authors have been attempting to develop more satisfactory air-sampling devices (Luckiesh *et al.*, 1946), with the result that two highly efficient devices have been developed. It is the purpose of this paper to describe them and show a few typical results.

RADIAL JET SAMPLER

Apparently one of the best samplers previously described is the slit sampler developed by Bourdillon, Lidwell, and Thomas (1941). Their publications and some private correspondence indicate that their apparatus, with some modifications not included in the original publication, would be valuable in studies of the value of different methods of air disinfection.

The authors, originally without knowledge of the work of Bourdillon and his colleagues, have developed the slit sampler (hereafter referred to as a radial jet sampler RJ) shown diagrammatically in figure 1. The air to be sampled is drawn through the sampler by means of a vacuum pump attached to the outlet. The air enters a closed metal drum, as shown, passes through a radial slit in the bottom of the drum, and impinges at high velocity on the culture medium in the petri dish below. It then passes over the edge of the dish and out through an opening below, as shown. At an air rate of 1 cubic foot per minute the velocity of air through the slit is approximately 100 feet per second. The petri dish is rotated at a rate of 2 revolutions per minute, to spread the colonies uniformly over the surface of the culture medium.

The efficiency of this sampler has been evaluated by connecting other samplers of a different type in series with it. Apparently the radial jet sampler collects more than 95 per cent of the air-borne bacteria.

The portability of this sampler is governed by the weight of the necessary vacuum pump and motor. This limits its usefulness in field tests as the authors have been unable to find a satisfactory lightweight vacuum pump and motor. It has very definite advantages otherwise if this difficulty can be overcome.

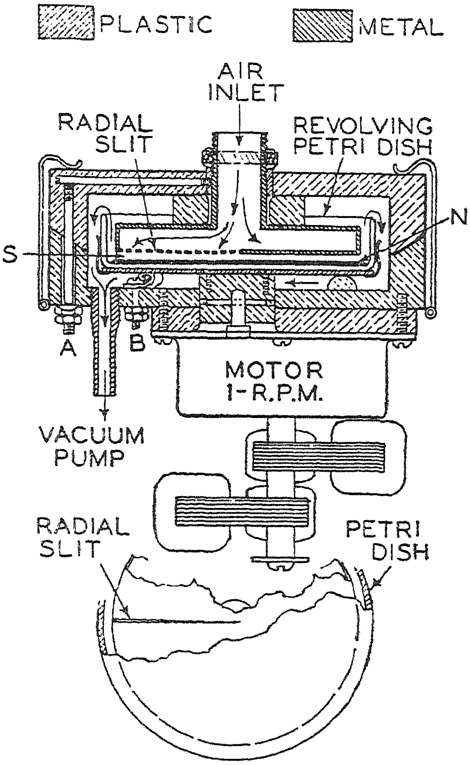

FIG. 1. A VERTICAL SECTION OF THE RADIAL JET AIR SAMPLER, RJ
At the bottom is a plan view of the radial slit in relation to the petri dish

ELECTROSTATIC AIR SAMPLERS

Since the electrostatic principle is effective in the collection of dust and smoke particles, the authors sought methods of applying this technique to the collection of bacteria. Not until their work was completed did they become aware of the development of an electrostatic sampler by Berry (1941).

In order to have a fairly constant source of air bacteria, a cabinet was constructed as illustrated diagrammatically in figure 2. To this was attached a small rectangular tunnel (figure 3), composed of a highly insulating material (lucite), through which air samples were drawn. This channel had metal

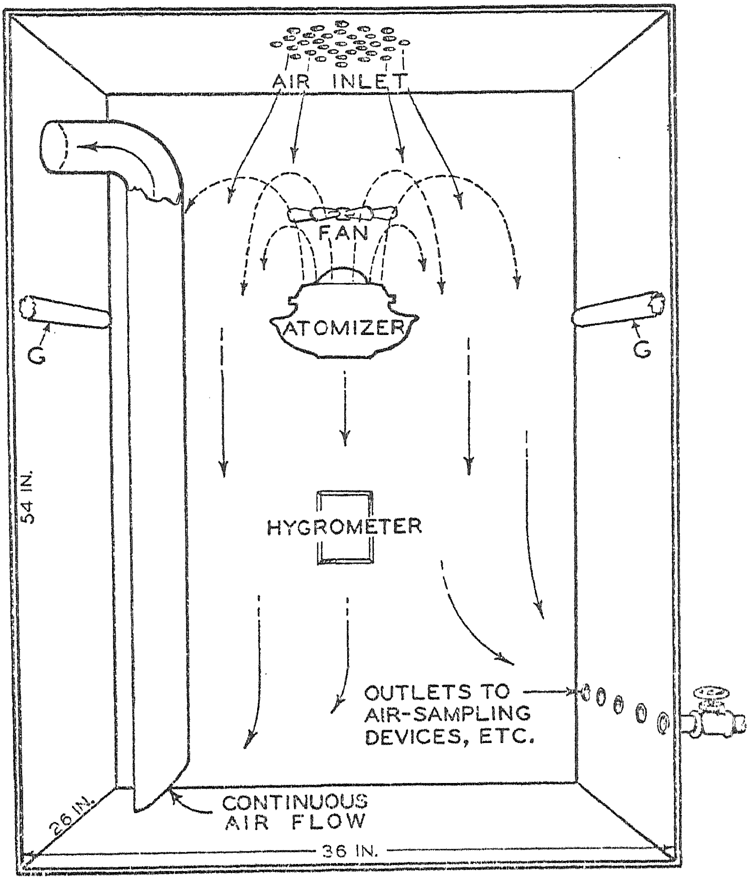

FIG. 2. AN INFECTION CHAMBER FOR ARTIFICIALLY INFECTING THE AIR WITH E. COLI BY MEANS OF AN ATOMIZER

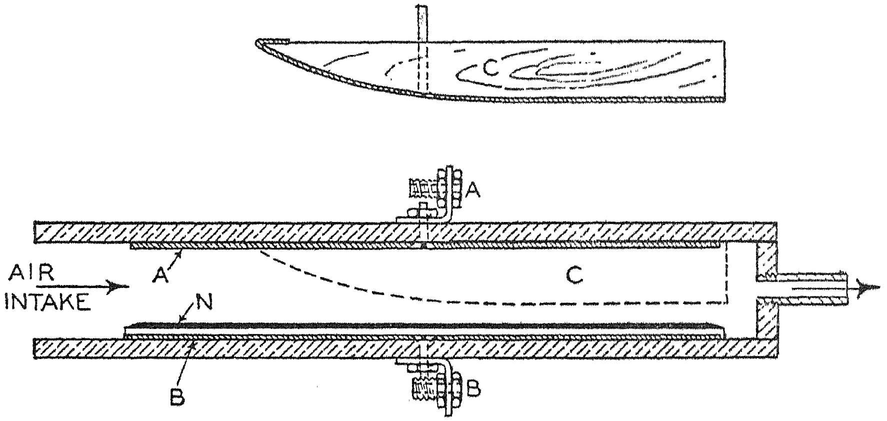

FIG. 3. A RECTANGULAR TUNNEL OF TRANSPARENT PLASTIC FOR STUDYING THE EFFECT OF AN ELECTROSTATIC FIELD IN BACTERIAL AIR SAMPLING

electrodes above and below. They were connected to a rectifier supplying direct current of any desired voltage from 0 to 8,000 volts. Either electrode could be made positive or negative. Also, the shape and spacing of the electrodes could be varied as desired by introducing elements such as *C*. A culture medium *N*, poured on rectangular flat plates, was used to collect the bacteria. *Escherichia coli* in broth was atomized into the chamber (figure 2) and the fan and continuous air flow through the chamber served to keep the concentration of organisms fairly uniform and constant.

It was immediately evident that an electrostatic charge greatly increased the collection of *E. coli*. The distribution of colonies along the length of the plate was greatly affected by the air rate and the applied voltage. It was found that if the upper electrode extended to a point immediately above the left-hand end of the collecting plate, many of the colonies were removed from the air by the electrical field before reaching the nutrient plate. When the electrode *C* was used, a much better collection resulted, the colonies being less concentrated at the left-hand end of the plate. By proper control of air flow and voltage, most of the *E. coli* entering the channel could be collected on the nutrient plate. In some cases nutrient plates were used at both top and bottom of the channel. Both plates collected *E. coli*, but the plate placed on the *positive* electrode collected nearly ten times as many as that on the negative electrode. Apparently, only static charges are involved, and it has been found that it is unnecessary to make electrical contact with the nutrient so long as the back of the plate is in contact with the electrode.

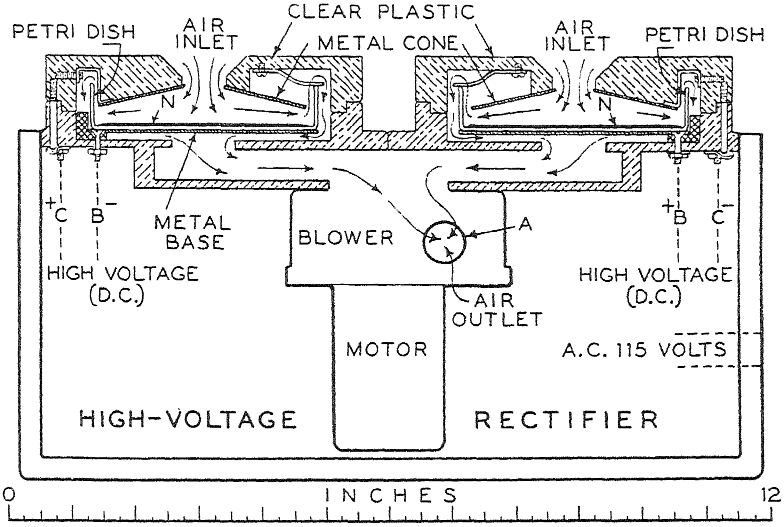

FIG. 4. A VERTICAL SECTION OF THE DUPLEX ELECTROSTATIC AIR SAMPLER, DE

The next step was to design and build a portable device employing the electrostatic principle. The first sampler employed a single petri dish, with a switch to reverse the polarities of the two electrodes when desired. Experience with this in collecting air-borne bacteria indicated the desirability of making simultaneous samplings with both positively and negatively charged electrodes under the petri dishes. The final result was the design and construction of the duplex electrostatic sampler DE shown in figures 4 and 5.

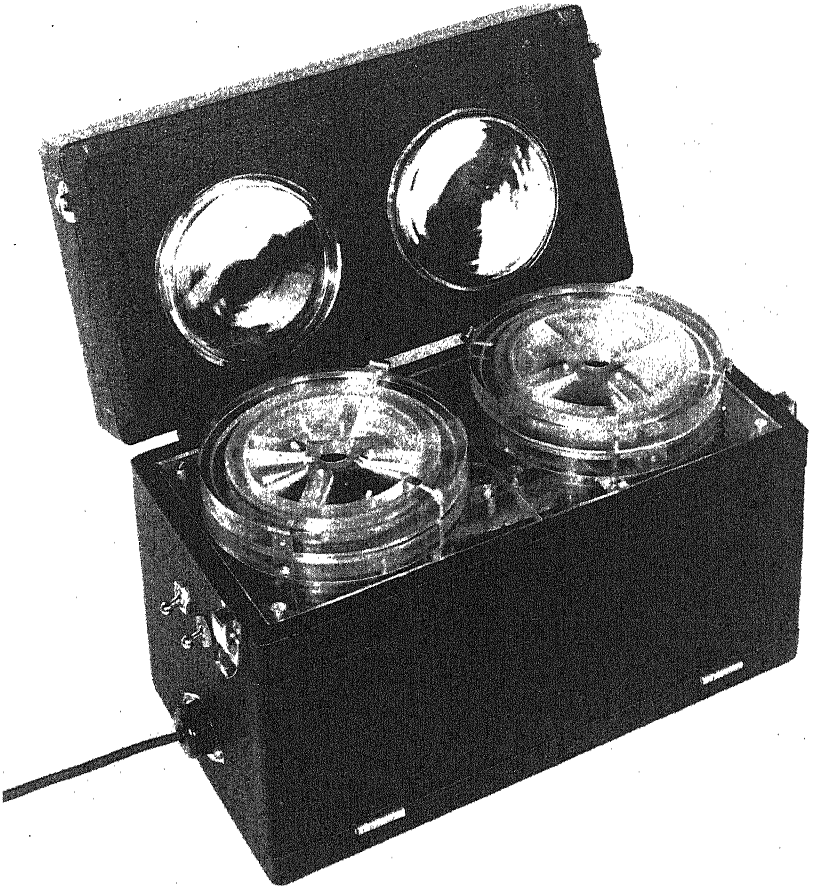

FIG. 5. EXTERNAL VIEW OF THE DUPLEX ELECTROSTATIC AIR SAMPLER, DE

As is shown in figure 4, the two petri dishes are placed in the two plastic units which have removable covers. A small electrically operated blower draws air at *equal rates* through the two units. One of these has the lower electrode negative and the upper electrode, a fairly flat metal cone, positive. In the other unit the electrical conditions are reversed. The applied voltage of approximately 7,000 volts is derived from a half-wave rectifier employing a high voltage transformer and an 879/2 x 2 rectifier tube. The air rate through each unit is 0.5 cu ft per minute. The shape of the upper electrode and the air rate were determined by many trials and have been chosen to give a good distribution of colonies over the plate area and a high efficiency of collection.

It is assumed, and experience verifies the assumption, that both positively and negatively charged bacteria exist simultaneously in the air. Those having a positive charge will be collected on the negative electrode (or the petri dish placed on it) and those negatively charged will be collected on the positive electrode. Consequently, one unit collects on the petri dish those positively charged and the other those which are negatively charged. Presumably, if a bacterium or the medium to which it is attached has no charge, it would not be selectively collected. Consequently, each unit collects on the petri dish both charged and uncharged bacteria. However, the partial duplication of catch in the two units is somewhat offset by the loss of some bacteria which pass through without collection in either unit. Quantitative results are discussed later.

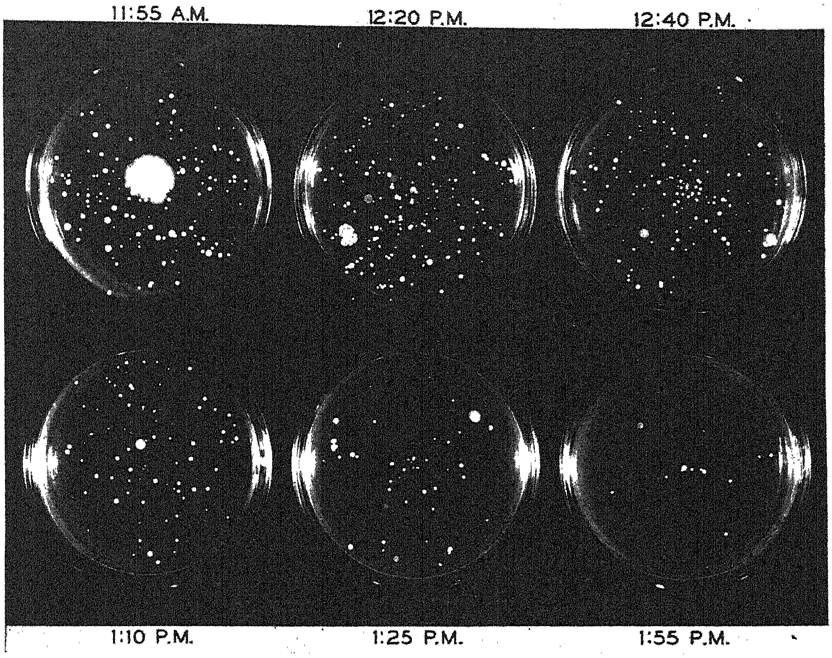

FIG. 6. PETRI DISHES SHOWING THE RESULTS OF SAMPLING AIR-BORNE BACTERIA IN A CAFETERIA DURING THE LUNCH PERIOD, NOVEMBER 12, 1945

The radial jet air sampler RJ was used at 1 cu ft per minute, with a total of 5 cu ft for each sampling. Largest crowd was in cafeteria at about 12:15 P.M.

In our work with naturally occurring air-borne bacteria, a tryptose blood agar base (Difco) was used. With this nutrient it is found that on the average there are approximately 30 per cent more colonies developed on the petri dish placed on the negative electrode than on that on the positive electrode when sampling naturally occurring air-borne bacteria. Whether or not this ratio is dependent upon the types of colonies which will grow on this medium, or whether it depends upon other factors, will be the subject of further studies. It is possible that the preponderance of negatively charged bacteria encountered in our tests with *E. coli* atomized into the air may be the result of charges imparted in the process of atomization. That both positively and negatively charged bacteria exist in the atomization chamber is evidenced by the fact that either a positive or a negative electrode collects more *E. coli* than one without charge.

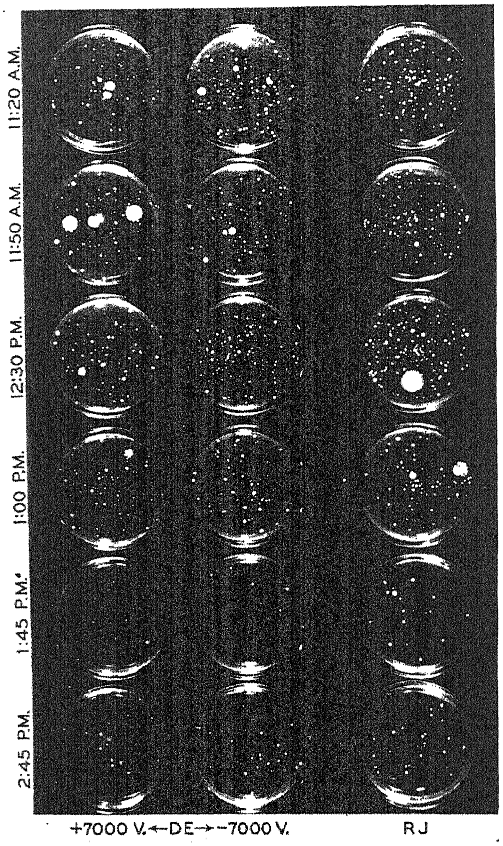

FIG. 7. PETRI DISHES SHOWING THE RESULTS OF SIMULTANEOUS SAMPLINGS OF THE AIR AT THE INDICATED TIMES IN THE SAME CAFETERIA

The duplex electrostatic sampler was used at +7,000 and –7,000 volts, and the radial jet sampler without voltage. Note that the sum of the colonies collected on the two dishes of the DE sampler is approximately the same as the number collected by the radial jet sampler.

In appraising the bacterial content of the air, our procedure is to assume that, when 5 cu ft of air are drawn through each unit of the duplex sampler, the *sum* of the colonies on the two petri dishes represents the total number of bacteria in 5 cu ft which will colonize on the medium used (neglecting the fact that this sampler may not break up clumps). Comparative tests with the radial jet sampler show that when the results are appraised in this way, the *average* collection by this duplex electrostatic sampler is approximately two or three per cent greater than that by the radial jet sampler when collecting naturally occurring air-borne bacteria.

Since the upper metal electrodes also collect bacteria, it was a question whether they should be sterilized between samplings to avoid contamination of subsequent samplings. In order to test this, air samples have been drawn from the atomization chamber (figure 2), subsequently followed by samplings of air sterilized by *massive irradiation* by germicidal energy. These tests indicated that it is unnecessary to sterilize the sampler, since the subsequent contamination was less than 0.1 per cent, regardless of whether or not the upper electrodes were cleaned with alcohol.

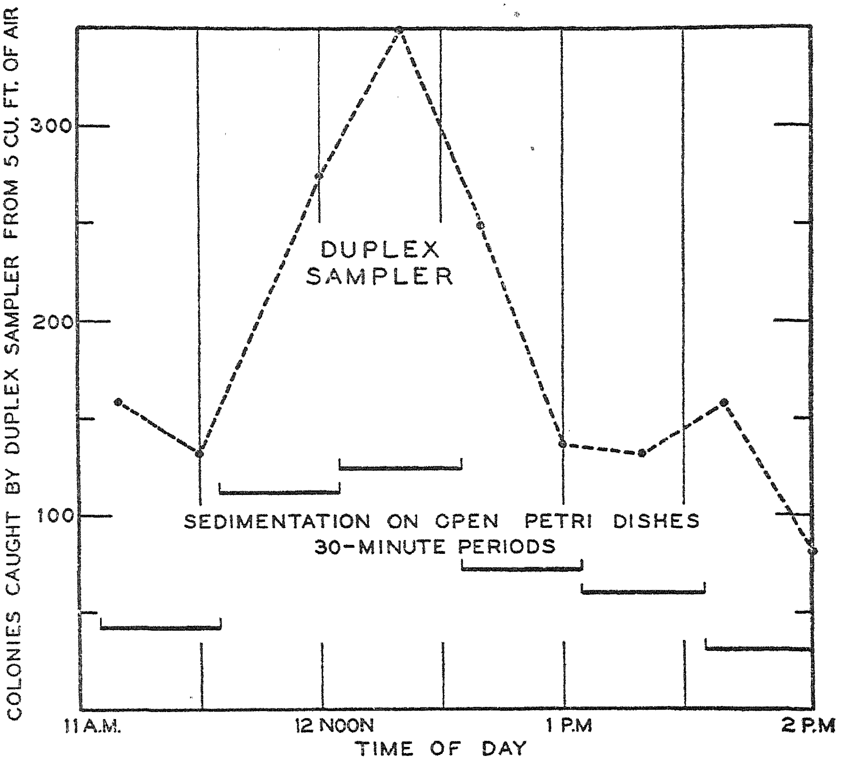

FIG. 8. A COMPARISON OF THE COLLECTION OF AIR-BORNE BACTERIA BY THE DE SAMPLER AND BY SIMPLY EXPOSED (SEDIMENTATION) DISHES

Figures 6 and 7 show some typical results obtained in sampling air in a cafeteria in November during the lunch period, which lasts from approximately 11:15 A.M. to 1:15 P.M. with the peak crowd at about the middle of this period. The petri dishes illustrated in figure 6 were exposed in the radial jet sampler. It will be noted that the colony distribution across the area of the dishes was very satisfactory.

For the results shown in figure 7 the DE and RJ samplers were operated simultaneously and in parallel in the same cafeteria, the air rate being 0.5 cu ft per minute through each unit of the DE sampler and 1 cu ft per minute through the RJ sampler. Each petri dish represents the sampling of 5 cu ft of air at the indicated time.

Figure 8 compares the bacterial content of the air in the cafeteria throughout the lunch period on a January day as appraised by the DE sampler and by sedimentation on open petri dishes exposed for 30-minute periods as indicated. The second slight peak may indicate activity in clearing the tables. Experience in all our tests indicates that many of the bacteria caught are from dust stirred up by activity.

In order to avoid any hazards due to the high voltage used in the sampler, 10 megohm resistors have been inserted between the terminals of the rectified voltage and the sampler electrodes. Although this sampler probably does not break up clumps or clusters of bacteria, this result might be achieved by collecting them in liquid, violently shaking the liquid in a test tube, then plating out.

As will be evident from figure 5, the duplex electrostatic sampler is very compact and portable. It weighs approximately 12 pounds complete, and the blower is sufficiently quiet to allow its use in public places. Many tests have been made in movie theaters, a cafeteria, etc., without disturbing the occupants.

The RJ sampler has also been tested with an electrostatic field, but this seems to be unnecessary unless air rates of the order of 0.5 cu ft or less per minute are used.

Our experience indicates that the duplex electrostatic sampler fulfills all the requirements set forth above, and we believe that it should prove to be a valuable tool for field tests. Much more information is needed to determine existing air contamination in many institutions and public places, and extensive use of such samplers should supply the necessary data.

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THE EFFECT OF STREPTOMYCIN ON PROTEUS INFECTIONS OF THE CHICK EMBRYO

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Streptomycin (Schatz, Bugie, and Waksman, 1944) has been shown to have a high *in vitro* activity against various species of *Proteus* (Robinson, Smith, and Graessle, 1944; Helmholz, 1945) and is efficacious in the treatment of some human infections (Herrell and Nichols, 1945). There are, however, no data on its activity in experimental *Proteus* infections other than the mere statement that streptomycin protects mice infected with *Proteus vulgaris*, (Jones, Metzger, Schatz, and Waksman, 1944). The present work is an attempt to determine the therapeutic effectiveness of streptomycin on *Proteus vulgaris* infections of the developing chick embryo.

METHODS

Fresh, fertile Leghorn eggs were incubated for 10 days in a standard egg hatcher and were prepared for use by creating an air space over the embryo. This was accomplished by drilling a small hole through the shell into the air space at the butt end of the egg and a second hole just through the shell and shell membrane on the side of the egg over the embryo. Suction was applied on the hole entering the air space until the chorio-allantoic membrane dropped away from the hole on the side of the egg. The two openings were covered with collodion and the embryos placed in a bacteriological incubator set at 37 C. The following day the 11-day-old embryos were candled for viability and infected by dropping 0.05 ml of a 10^7 dilution of a 24-hour peptone water culture of *Proteus vulgaris* on the chorio-allantoic membrane. This inoculum was approximately the minimum infecting dose and contained between 10 and 150 organisms, as determined by plate counts. The streptomycin[1] was diluted to contain the proper amount in 0.1 ml saline, and was applied as was the inoculum. The strain of *Proteus vulgaris* used in these studies was one from our stock culture collection which had been isolated from a human source less than three years previously. *In vitro* bacteriostatic tests showed that growth was inhibited in peptone water at a concentration of 1.8 units per ml. The infected and treated embryos were maintained in a 37 C bacteriological incubator and candled daily to determine viability. They were autopsied at death, or, if still alive, they were sacrificed on the seventh day following infection. Cultures were routinely made of the chorio-allantoic membrane and of the amnionic fluid. Cultures were also occasionally made of the blood or of the various organs. A semiquantitative procedure was used in making cultures from the chorio-allantoic membrane and the amnionic fluid. Green's method (Green and Birkeland, 1944) utilizing a swab to pick up organisms from the chorio-allantoic membrane and streaking on one-half of an agar plate was employed to estimate the degree of infection. To determine approximately the number of organisms in the amnionic fluid, the amnionic sac was carefully opened and the tip of a dry swab inserted and then streaked on the other half of the agar plate. The growth on the two halves of the plate served as an index of the number of organisms present in the two parts of the embryo.

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[1] The streptomycin hydrochloride used in these studies was kindly supplied by Dr. D. F. Robertson of Merck and Co., Inc.

EXPERIMENTAL

Proteus vulgaris produces a rapidly advancing generalized infection in 11-day-old chick embryos, and death occurs within 48 hours following infection (table 1). At autopsy the exposed chorio-allantoic membrane is covered with a thick gray plaque and the embryo is extremely hemorrhagic. The organism can be abundantly recovered from the plaque on the chorio-allantoic membrane, the amnionic fluid, the blood, and the various organs of the embryo. The hemorrhagic appearance and the characteristic fecal odor of embryos dying from *Proteus* infection strikingly separate them from embryos dying of natural causes.

TABLE 1

Effect of administration of streptomycin 18 hours following infection

UNITS OF STREPTOMYCIN	NUMBER OF EMBRYOS	SURVIVAL OF EMBRYOS IN DAYS							NO. OF EMBRYOS GIVING POSITIVE CULTURE	MEAN SURVIVAL TIME (DAYS)
		1	2	3	4	5	6	7		
None (controls)	23	22	6	2	0				23	1.3
50	8	8	2	1	0				8	1.3
250	8	8	3	2	2	1	1	0	8	2.1
500	10	9	2	0					10	1.1
1,000	5	5	5	5	5	4	3	3	5	6.0+*

* The + sign indicates that the embryos alive on the seventh day were sacrificed, and hence the mean survival time is greater than the value indicated.

In the first series of experiments treatment with streptomycin was begun 18 to 20 hours following infection (table 1). At this stage the infection is well advanced and is grossly indicated by the plaque on the chorio-allantoic membrane and by the reduced activity of the embryo as evidenced by candling. The mean survival time of the untreated embryos was 1.3 days and was extended to 6.0+ days when the infected embryos received 1,000 units of streptomycin. Smaller amounts of streptomycin were ineffectual in arresting these advanced infections. Embryos receiving 500 units or less all died of *Proteus* infection. The organism was readily recovered from these embryos, and the dead embryos had all the characteristics of death due to *Proteus vulgaris*. *Proteus vulgaris* could also be recovered from the embryos receiving 1,000 units. In this series, 2 of the 5 embryos were sacrificed before they died because of improper candling.

The remaining 3 embryos were sacrificed on the seventh day. These 5 embryos when examined had a normal healthy appearance and the fecal odor was absent. This suggested that 1,000 units of streptomycin did not free the embryo of *Proteus vulgaris* but suppressed the effects of the organism until the embryo became resistant to it. In older embryos the organism could exist and perhaps multiply somewhat, but the embryo would be resistant to its effects. To verify this possibility, five 16-day-old embryos were infected with approximately 100 organisms, and five additional embryos were infected with 10,000 organisms. These embryos were sacrificed just before hatching (5 days later) and cultured for *Proteus vulgaris*. All were alive and appeared to be normal and healthy, but confluent growth of *Proteus vulgaris* was obtained when swabs from the chorio-allantoic membrane and the amnionic sac were streaked on agar plates. It would seem then that larger or repeated doses are required to eliminate the organism from the embryo when treatment is begun at this advanced stage of infection.

TABLE 2

Effect of administration of streptomycin 6 hours following infection

UNITS OF STREPTOMYCIN	NUMBER OF EMBRYOS	SURVIVAL OF EMBRYOS IN DAYS							NO. OF EMBRYOS GIVING POSITIVE CULTURE	MEAN SURVIVAL TIME (DAYS)
		1	2	3	4	5	6	7		
None (controls)	23	22	6	2	0				23	1.3
50	5	4	4	4	3	2	2	2	5	4.2+
250	5	4	3	3	3	3	3	3	3	4.4+
1,000	5	5	5	5	5	4	4	4	1	6.2+

In the second series of experiments treatment with streptomycin was begun 6 hours following infection (table 2), as it was felt that 18-hour infections were so advanced that abnormally high concentrations of streptomycin were needed to show any effect. The mean survival time of embryos receiving 50 units streptomycin was extended to 4.2+ days. *Proteus vulgaris* was recovered from all the embryos. However, the two sacrificed on the seventh day lacked the gross characteristics of *Proteus* infection. The mean survival time of the embryos receiving 250 units was about the same as those receiving 50 units. In this group *Proteus vulgaris* was recovered from only 3 embryos, the 2 dying early and 1 of the 3 sacrificed on the seventh day. Embryos receiving 1,000 units 6 hours after infection were well protected and the organisms eliminated, with the exception that one of the embryos sacrificed on the seventh day yielded a few organisms from the chorio-allantoic membrane. The embryo dying on the fifth day died from other causes as no organisms could be isolated from any part of this embryo.

The investigation was further extended by a series of experiments in which the streptomycin was administered in 3 treatments (table 3). The embryos received treatments at 6, 30, and 54 hours following infection. Thirty units in 3 treatments were approximately as effective as 50 units in 1 treatment.

One hundred and fifty units in 3 treatments were more effective than 250 units in 1 treatment. Four of the five embryos receiving 150 units in 3 treatments were devoid of organisms, whereas only 2 of the 5 embryos receiving 250 units in 1 treatment were free of organisms. *Proteus vulgaris* was recovered from only 1 of the 2 embryos dying on the fourth day (table 3), the second embryo dying from natural causes. These doses are in accord with the 150 to 300 units that Jones, Schatz, and Waksman (1944) used to protect embryos infected with *Shigella gallinarum* and *Brucella abortus*. *Proteus vulgaris* was completely eliminated from the embryos receiving 750 units in 3 treatments, and the mean survival time was extended to 6.2+ days.

TABLE 3

Effect of administration of streptomycin 6, 30, and 54 hours following infection

UNITS OF STREPTOMYCIN		NUMBER OF EMBRYOS	SURVIVAL OF EMBRYOS IN DAYS							NO. OF EMBRYOS GIVING POSITIVE CULTURE	MEAN SURVIVAL TIME (DAYS)
Per treatment	Total		1	2	3	4	5	6	7		
None	0	23	22	6	2	0				23	1.3
10	30	5	4	3	3	3	2	2	2	4	3.8+
50	150	5	5	5	5	3	3	3	3	1	5.4+
250	750	5	5	5	5	5	4	4	4	0	6.2+

SUMMARY

Proteus vulgaris produces a rapidly fatal infection in 11-day-old chick embryos, whereas those 16 days old are relatively resistant.

Streptomycin in doses of 1,000 units or more is necessary to arrest 18-hour infections. The drug is considerably more effective when administered 6 hours after infection. Two hundred and fifty units in 1 dose or 150 units in 3 daily doses served to protect approximately 50 per cent of the embryos. Larger doses when administered early not only gave protection but in addition eliminated the organism from the embryo.

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STUDIES ON THE DEATH OF BACTERIA AT LOW TEMPERATURES

II. The Comparative Effects of Crystallization, Vitromelting, and Devitrification on the Mortality of Escherichia coli

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In a previous paper Weiser and Osterud (1945) presented evidence that the death of bacteria by freezing involves a rapid-acting or "immediate" death, caused by freezing and thawing per se, and a "storage" death, which is a direct function of time and temperature. Evidence was also presented which supported the theory that immediate death results principally from the mechanical action of extracellular ice. The present investigation is an attempt to gain additional information concerning the mechanism of death of bacteria by freezing.

LITERATURE

When water is cooled to temperatures below 0 C, under ordinary circumstances it crystallizes to form ice. However, if cooled to very low temperatures under special conditions, it may solidify without crystallizing, a state analogous to glass, termed vitreous. The vitrification of pure water is very difficult to accomplish but has been reported by Hawkes (1929) and by Burton and Oliver (1935).

Any extensive discussion of the factors influencing the crystallization, vitrification, and devitrification of water is not within the scope of this article. They are well treated in such works as those of Luyet and Gehenio (1940) and Dorsey (1940). Briefly, vitrification of any aqueous solution can only be accomplished by reducing the temperature through the zone at which crystallization occurs so rapidly that there is insufficient time for crystals to form. With ordinary water the velocity of the formation of ice crystal nuclei and of crystal growth is so great that vitrification is seldom accomplished. However, the inclusion in water of substances such as sucrose or gelatin, which increase its viscosity, greatly retards the orientation of water molecules to form ice crystals and thus makes vitrification easier to accomplish. Vitrification of these solutions can usually be effected by subjecting them, in very thin films, to low temperatures such as that of liquid nitrogen. If vitreous water is warmed slowly, devitrification will occur, whereas if it is warmed rapidly vitromelting will occur. Slow warming does not result in a change to the crystalline state (devitrification) until temperatures approaching 0 C are reached. For example, in the case of vitrified 2 M sucrose the temperature at which devitrification takes place has been reported by Luyet and Gehenio (1940) to be −31.8 C. Devitrification can be completely avoided and the vitreous water converted directly to liquid provided the temperature is raised through the crystallization range very rapidly, a process called vitromelting.

Stiles (1930) upheld the theory that cell death from freezing is due to changes in the physical structure of the protoplasm brought about by the formation of ice crystals within the cell. He suggested that the colloids of protoplasm may be able to withstand vitrification and vitromelting better than freezing and thawing. As evidence of this he presented his finding that hydrosols of pure chlorophyll and of gum mastic are less altered by rapid cooling to very low temperatures than by slow cooling to the same temperatures. Whether vitrification was actually observed in the materials which were cooled rapidly was not stated. The author did not extend his investigations on vitrification to include microorganisms.

Luyet was the first to investigate the vitrification of living cells. The results of the studies of Luyet and his co-workers extending from 1937 to May, 1939, are summarized by Luyet and Gehenio (1940) in their book *Life and Death at Low Temperatures*. In their preliminary work these investigators used a number of materials including gelatin, sugars, albumin, gums, dextrin, glycerine, and sodium chloride. Solutions of these substances were spread in thin films on cover slips, etc., and their reactions to treatments favoring vitrification, devitrification, and vitromelting were studied. The investigations were then extended to include living cells.

Luyet and Gehenio were not certain that vitrification of the protoplasm of living cells actually occurred. However, the likelihood that it took place was considered great. They pointed out that proof that fewer organisms are killed under the conditions favoring vitrification than under similar low temperature treatments which produce crystallization would constitute indirect evidence that vitrification of protoplasm had been achieved. They stated that very small organisms below one-third of a mm in thickness should be capable of vitrifying provided their water content does not exceed 90 per cent. In their attempt to develop successful methods for producing vitrification, they tried many different suspending mediums, methods for obtaining thin films, and procedures for accomplishing rapid cooling of films. Films prepared on thin sheets of mica and in fine wire loops were found to be the most satisfactory.

These authors subjected various members of the rhizopods, the ciliates, and the flagellates to vitromelting treatment. Failure to revive a single one of these organisms led them to conclude that the organisms had not undergone vitrification because of too high a water content. In subsequent trials a small percentage of myxamoebae and frog spermatozoa were reported to survive vitromelting treatment, whereas rat spermatozoa were all killed. Plasmolyzed cells from moss and onion epidermis survived, as judged by their ability to deplasmolyze following treatment. Treated fibers of frog muscles retained only limited power of contraction. The authors summarized their experiments by saying, "Although there are several unexplained exceptions, the results obtained in the vitrification of protoplasm, and in particular those furnished by moss, epidermal cells and muscle fibers, seem to confirm the view that a good vitrification is not injurious, there being no molecular disturbance, while an incomplete vitrification or devitrification and, *a fortiori*, crystallization, are injurious to the extent that they disrupt the living structure."

Alexander Goetz and S. Scott Goetz (1938) and Alexander Goetz (1939) also regarded freezing injury as a physical death due to the formation of intracellular ice. They stated, "In contradistinction to crystallization, vitrification would not disturb the molecular arrangement of the cell contents, and also, the more macroscopic effect of crystallization, the lesion of the cell membrane, is impossible." They compared the effects of crystallization and vitromelting on yeast cells suspended in Ringer's solution and in gelatin. Cooling baths of liquid air and liquid nitrogen were used. The vitrification procedure consisted of immersing thin films of a yeast suspension (held in a fine platinum loop) in cooled isopentane. Melting of the vitrified material was effected by rapid transfer to a bath of petroleum ether at room temperature. As a control, crystallization was produced by immersing test tubes containing larger quantities of the suspension in the freezing bath. The methylene blue differential staining method was used for determining the numbers of living and dead cells. The authors reported that the mortality resulting from freezing and thawing was approximately 75 per cent, whereas that resulting from the vitromelting treatment was approximately 10 per cent. The death rate which occurred during devitrification treatment was observed to increase gradually over the devitrification temperature range of −140 C to −5 C, becoming very appreciable at the higher temperatures.

Breedis (1942) has reported that the vitromelting treatment completely inactivates the cells of mouse leukemia, whereas slow freezing to the same temperature permits many to remain viable.

Reports on the effects of crystallization, vitromelting, and devitrification treatments on cells are few, and final unequivocal evidence on the comparative influence of these treatments will not be complete until additional experiments are conducted in which the time-temperature changes of controls parallel the vitromelting treatment closely. Moreover, the methods of determining the viability of cells must be more certain than those which have been generally employed so far.

GENERAL METHODS

The methods for culturing organisms, preparing suspensions, determining numbers of organisms, and analysis of data, etc., were in most respects similar to those previously used by Weiser and Osterud (1945).

The stock suspensions of bacteria used in the present experiments were prepared as follows: The organisms were collected from agar slopes in M/60 phosphate buffer, pH 7.0, centrifuged, and resuspended in a small volume of buffer. An aliquot of this was diluted and the number of organisms estimated using the Klett-Summerson photoelectric colorimeter. The stock suspension was then prepared by diluting with 10 per cent cp sucrose. This dilution was usually about 1:100, so that changes in the final concentration of sucrose were negligible. The inclusion of 10 per cent sucrose was found to facilitate vitrification. The stock suspensions were stored at 8 C to 10 C during the course of the experiments in order to keep the numbers of viable organisms as constant as possible. Dilutions for plating were made in M/60 phosphate buffer, pH 7.0, and the platings were conducted in quadruplicate. Incubation of plates was continued for at least 3 days at 37 C.

For the preparation of films very small drops of approximately 0.02 ml (larger drops result in thick films which will not vitrify) were measured with a fine, calibrated capillary pipette from the stock sucrose suspension and placed between two sterile, no. 1, 22-mm cover slips. The cover slips were pressed together to aid capillary action and secure thin films. The preparations were handled with sterile instruments and suspended in sterile, fine-wire baskets during treatment. The container used for the liquid nitrogen was a pint thermos bottle.

The crystallization treatment consisted of holding the film preparations in the atmosphere above the liquid nitrogen until freezing was observed (about 3 seconds) and then immersing them in the liquid nitrogen for 30 seconds. Following this they were removed from the bath, allowed to remain in the air until thawed (which occurred in about 15 seconds), and promptly diluted and plated. The dilution bottles were observed while being shaken for several minutes to make certain that the slips became separated so as to allow proper dispersal of the organisms.

Vitromelting treatment was conducted by quickly plunging prepared films into liquid nitrogen. The preparations usually reached the temperature of liquid nitrogen in about 2 seconds, as evidenced by the cessation of bubbling. Thirty seconds later they were rapidly transferred to the diluting buffer at room temperature. Usually the preparations were typically vitreous in appearance when removed from the nitrogen bath. The few which showed evidence of crystallization were discarded. The melting of the films took place quickly in the diluting buffer without any visible evidence of crystallization.

The devitrification treatment was accomplished by first vitrifying the films in liquid nitrogen and then warming them to room temperature in the air. The following changes were noted in these preparations: Upon removal from the liquid nitrogen they were transparent, indicating that vitrification had occurred. A light cloud then appeared, because of the condensation of atmospheric moisture on the outside of the slips. This was quickly followed by the formation of a heavy cloud resulting from the freezing of this condensed moisture. With further warming a still heavier cloud gradually appeared, which represented the formation of ice crystals (devitrification) in the material between the slips. The last visible change to occur was the melting, which always began at the periphery of the sample. These changes were all complete in about 30 seconds after the removal of the preparations from the nitrogen bath. The results following the various treatments are given as the average percentage of reduction and range of reduction in plate count calculated from the means. The standard deviation of means (σm) and coefficient of variation (C_v) of plate counts are also given.

RESULTS

The influence of crystallization, vitromelting, and devitrification on the mortality of Escherichia coli. The first experiment was designed to compare the lethal effects of vitromelting and devitrification. A heavy, washed suspension of *E. coli* was prepared in M/60 phosphate buffer from 19-hour agar slope cultures and diluted with 10 per cent sucrose solution to an estimated 6,000,000 organisms per ml. This stock suspension was stored at 8 C to 10 C for 2½ hours before the beginning of the experiment. Storage was continued at this temperature during the course of the experiment.

Four untreated cover slip preparations made from the stock suspension were diluted and plated in quadruplicate at the beginning of the experiment. Four similar untreated control samples were plated at the termination of the experiment. Additional controls consisted of 4 crystallized samples and 4 samples prepared from sterile 10 per cent sucrose. The samples prepared from sterile sucrose were vitrified by immersion directly in liquid nitrogen, devitrified at room temperature in the air, diluted in 5 ml of sterile buffer, and plated in 2.5-ml amounts in each of 2 petri dishes. They were employed to detect contamination originating from either the liquid nitrogen or other sources.

TABLE 1

The influence of vitromelting and devitrification on the percentage of reduction in plate counts of E. coli

Suspension of 19.0-hour culture in 10 per cent sucrose

TREATMENT	AVERAGE REDUCTION	RANGE OF REDUCTION	σ m	C_v
Vitromelted	48.6	45.3–51.7	4.00	6.40
Devitrified	55.1	50.4–59.4	9.80	17.90

The treated preparations consisted of 9 vitromelted samples and 9 devitrified samples. The order of the experiment was as follows: The untreated controls were plated at the beginning of the experiment, followed in rapid succession by the alternate treating and plating of pairs of vitromelted and devitrified samples. Then stored untreated controls, contaminant controls, and crystallized controls were plated in rapid succession. No apparent reduction of viable organisms occurred in the stored controls during the course of the experiment. The results are given in table 1.

The initial untreated control counts showed a remarkably good correlation between samples, the range being 16,887 to 18,715, σm 31.67, and C_v 6.6. These counts were used as a basis for calculating the percentage of mortality of treated samples. The controls subjected to crystallization treatment showed an average reduction in plate count of 30.4 per cent. The range of reduction was 25.1 per cent to 34.9 per cent, σm 14.80, and C_v 11.6. The counts on contaminant control samples indicated that no appreciable contamination had occurred. The count averaged only 8 organisms per sample, which is insignificant in comparison with the numbers of organisms which survived the various treatments. The differences in mortality between the treated samples were statistically significant, and clearly established that the devitrification treatment was more lethal than the vitromelting treatment. A comparison of the results of these treatments with those of the crystallization treatment indicated that mortality is lowest following crystallization, a finding which was confirmed in the second experiment.

A second experiment similar to the first was planned to compare the lethal effects of crystallization, vitromelting, and devitrification. A heavy, washed suspension of *E. coli* was prepared in M/60 phosphate buffer, pH 7.0, from 20-hour agar slope cultures and diluted in 10 per cent sucrose solution to an estimated 10,000,000 organisms per ml. This stock suspension was stored at 8 C to 10 C and the experiment begun 10 minutes later.

Four untreated control cover slip preparations from the stock suspension were diluted and plated in quadruplicate at the beginning of the experiment. Four additional untreated control samples were plated at the termination of the experiment 2 hours later. Controls were also included to detect contamination as in the preceding experiment.

TABLE 2

The influence of crystallization, vitromelting, and devitrification on the percentage of reduction in plate counts of E. coli

Suspension of 20.0-hour culture in 10 per cent sucrose

TREATMENT	AVERAGE REDUCTION	RANGE OF REDUCTION	σ m	C_v
Crystallized	33.00	30.48–35.49	26.40	11.72
Vitromelted	61.51	57.70–65.28	39.87	26.10
Devitrified	82.75	80.47–84.67	20.50	35.53

The treated preparations consisted of 10 crystallized samples, 10 devitrified samples, and 12 vitromelted samples. They were treated and plated, two at a time, in rapid succession in an orderly alternating sequence so as to correct for any reduction in numbers of viable organisms and possible changes in resistance taking place in the stock suspension during the course of the experiment. The results are presented in table 2.

A small but significant reduction in numbers of viable organisms in the stock suspension took place during the course of the experiment. The average initial count was 2,320.8, the range 2,191.8 to 2,449.8, σm 64.50, and C_v 20.56. The average final count was 1,900, the range 1,787.6 to 2,012.4, σm 56.20, and C_v 10.94. The mean of these means, or 2,110.4, was used for computing the percentage of reduction in plate count for the treated samples. The controls plated to detect contamination were negative.

The comparative effects of the various treatments were similar to those of the first experiment. The devitrification treatment resulted in a mortality of 82.75 per cent, the vitromelting treatment in a mortality of 61.51 per cent, and the crystallization treatment in a mortality of 33.00 per cent.

A comparison of this experiment with the first experiment reveals that, although the mortalities in the crystallized samples of the two experiments are not significantly different, those of the vitromelted and devitrified samples are much greater in the second experiment than in the first. It is probable that the difference of 1 hour in the age of the cultures used in the two experiments is not responsible for this disparity in the results. The only apparent difference in the manner of conducting the experiments which could reasonably account for the result was the period of storage of the stock sucrose suspension before treatment. In the first experiment the stock suspension was stored for 2½ hours before the beginning of the experiment, whereas in the second experiment the preliminary storage period was 10 minutes. The most obvious interpretation is that storage in 10 per cent sucrose enhances the ability of the organisms to survive devitrification and the vitromelting treatment but not crystallization treatment. The truth of this interpretation can only be determined by further experiments expressly designed to test the point.

DISCUSSION

The conditions of time and temperature employed in the crystallization and vitromelting treatments were very similar. Hence it is quite certain that the marked disparity in mortality observed was due to a difference in the physical states of water effected by the two treatments. Any attempt to fit the results of the present experiments to theories of the mechanism of death of bacteria by freezing involves considerable speculation, concerned for the most part with the states of water obtained.

Evidence presented by Weiser and Osterud (1945) indicates that ice does not form within bacterial cells during low temperature treatments. The only alternative state of water at extremely low temperatures (even in partially dehydrated cells) is the vitreous state, since water cannot exist as a liquid at these temperatures.

In the case of the vitromelting treatment it can be safely assumed that vitrification and the subsequent melting of the suspending medium took place without the formation of any appreciable quantity of extracellular ice. Nevertheless, the mortality resulting from this treatment was much greater than that following the crystallization treatment, in which the formation of extracellular ice is the principal cause of death. The most obvious interpretation of this finding is that, either the direct effect of vitrification of the suspending medium is more lethal than is crystallization, or the intracellular physical state effected by vitromelting is more injurious than that effected by the crystallization treatment. Of these the latter appears to be the more likely.

In the case of the crystallization treatment it is probable that the partial dehydration attending extracellular crystallization renders cells resistant to adverse effects accompanying further reductions in temperature, particularly to intracellular vitrification. This is indicated by the observation of Weiser and Osterud (1945) that in preparations which are frozen at low temperatures "immediate" death due to freezing is uniform for all temperatures, including those above the vitrification range; and, also, by the finding that repeated fluctuation of temperature of frozen suspensions through the range of −2 C to −195 C (a range at which repeated vitromelting should occur) does not result in greater death than that observed in stored control samples.

Of singular interest in this regard is the observation of Breedis (1942) that tumor cells of mouse leukemia when cooled at different rates to −196 C survive slow freezing better than rapid freezing. It is probable that effective dehydration of these large cells takes place only when the freezing process is relatively slow. Such partially dehydrated cells apparently become resistant to the adverse effects of further cooling to extremely low temperatures. Whether the adverse effects in this instance include the reaction of intracellular crystallization or vitrification, or both, is uncertain, since there is no evidence that ice does not form in large cells at extremely low temperatures. In any event, protection should be afforded by dehydration, since the amount of water available for either crystallization or vitrification would be limited. In the light of these remarks we suggest that the following events take place in the treatments employed in the present experiments:

In the crystallization treatment the first significant change to occur is the formation of ice in the suspending medium. This process exerts mechanical pressure, concentrates materials in the intercrystallic film, and brings about a partial dehydration of the cells. Since bacterial cells are small, the loss of water probably takes place with such great speed that, no matter how rapidly the temperature is lowered, the resulting dehydration is sufficient to increase the resistance of the cell to the adverse effects attending further cooling, particularly intracellular vitrification. During the period of crystallization of extracellular ice, death of a number of cells takes place as the result of the mechanical action of ice crystals and the concentration of solutes in the intercrystallic film. When the temperature reaches the vitrification point (about −110 C), vitrification of intracellular water occurs but does not produce injury because the cells have become partially dehydrated during the earlier crystallization of extracellular water.

In the case of the vitromelting treatment extracellular ice does not form in appreciable amounts in the first stages of cooling. Consequently, dehydration of the cells does not occur. Upon further cooling to the vitrification level vitrification of both extracellular and intracellular water takes place with attending injury. Whether this injury results from the vitrification of extracellular water or of intracellular water or of both cannot be ascertained from existing data.

The devitrification treatment presumably includes both the injury of vitrification upon cooling and the injury of crystallization upon warming. The summation effect of these two influences could well account for the high mortality observed following this treatment. However, this conclusion lacks certainty because the time-temperature relations in devitrification did not parallel the other treatments closely.

SUMMARY

Escherichia coli was suspended in 10 per cent sucrose and subjected to crystallization, vitromelting, and devitrification treatments at −195 C. The vitromelting treatment was found to be more lethal than the crystallization treatment.

The devitrification treatment was more lethal than either the vitromelting or the crystallization treatment.

The manner in which these results may apply to theories of the mechanism of the death of bacteria by freezing is discussed.

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THE RELATIVE ACTIVITY OF PENICILLINS F, G, K, AND X AGAINST SPIROCHETES AND STREPTOCOCCI IN VITRO

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The therapeutic use of penicillin has been complicated by the demonstration (Chemistry of Penicillin, 1945) that there are at least four molecular species, identified as F, G, K, and X, the relative proportions of which in the commercial product may vary with different strains of mold and different methods of concentration, and even in the output of a single plant at different times. Against a standard test strain of *Staphylococcus aureus*, the activities of penicillins F, G, K, and X have been found to be, respectively, 1,550, 1,667, 2,300, and 900 units per mg (Veldee *et al.*, 1945; Coghill, 1946; Libby and Holmberg, 1945; Welch *et al.*, 1944). This corresponds to relative gravimetric activities against the same organism of approximately 90, 100, 140, and 55. The relative molar activities are of the same order of magnitude.

In the case of penicillins G and X these relative activities have been shown to vary from organism to organism. Thus, per staphylococcidal "unit," Welch, Putnam, Randall, and Herwick (1944) found X to be significantly more active than G against gonococci in man and 3 to 5 times more active against pneumococci (I) in white mice; Libby and Holmberg (1945) found X to be gravimetrically 1.2 to 2 times more active than G *in vitro* against 3 strains each of pneumococci (I, II, III) and streptococci (A, B, D); and Ory, Meads, and Finland (1945) found 2 preparations in which X constituted 65 and 90 per cent of the total penicillin to be 2 to 8 times more active per unit than G against most strains of group A streptococci, gonococci, and meningococci *in vitro*, and twice as active against most strains of pneumococci and *Streptococcus viridans*.

The present communication will deal with the relative activities *in vitro* of penicillins F, G, K, and X against a group A hemolytic streptococcus (strain C-203) and a cultured strain of *Spirochaeta pallida* (Reiter).

METHODS AND MATERIALS

Penicillins. The nine lots of crystalline penicillin used in the present study are listed in table 1. The courtesy of several pharmaceutical houses, of Dr. R. D. Coghill, then of the Northern Regional Laboratories of the Department of Agriculture at Peoria, Illinois, and of Dr. D. C. Grove of the Food and Drug Administration in providing these specimens is gratefully acknowledged.

Weighed samples were dissolved in 0.85 per cent NaCl, sterilized by Seitz filtration, and tested by the techniques described below. At first the solutions were Seitz-filtered in 1:10,000 concentration; but it was found that at that concentration the major portion of the penicillin might be adsorbed by the filter pad. It is obvious that a large error may be so introduced in the assay of urine or serum specimens containing barely demonstrable concentrations of penicillin (e.g., 0.15 units per ml, or a 1:33,000,000 dilution of penicillin G). In the present experiments an attempt was made to minimize this adsorption error by filtering 10 to 15 ml of the solutions in 1:1,000 concentration and discarding the first third of the filtrate. Under such conditions the degree of adsorption was usually of the order of 0 to 10 per cent, did not significantly vary among the several types of penicillin, and would therefore cancel out in comparing the relative activity of a series of penicillins similarly filtered. This was confirmed by simultaneous assays of a series of penicillin solutions (a) sterilized by filtration and (b) prepared aseptically and tested without filtration, using a sufficiently heavy inoculum of streptococci to overgrow the chance contaminant. As is indicated in table 1, the assays of relative activity were similar in such parallel experiments. In later experiments, the solutions were sterilized by filtration through sintered glass discs, on which penicillin was not demonstrably adsorbed.

TABLE 1

Samples of crystalline penicillin used in present study

TYPE OF PENICILLIN	PRODUCER	PRODUCER'S LOT NUMBER	IDENTIFYING NUMBER IN TABLES
F	Abbott	PT433	I
	Upjohn	175-EANW-6	II
G	Squibb	CRA2N-20	I
	Upjohn	45ANW7	II
	Dept. Agric.	1724-22-B	III
K	Abbott	RP309-P1	I
X	Lederle	CA(32-42)IC	I
	Dept. Agric.	1624-43-A	II
	Food & Drug Administration		III

Streptococcidal assay. The method used for the assay of streptococcidal activity was a modification of those described by Rammelkamp (1942) and Kirby and Rantz (1944). The stock penicillin solution was diluted in beef infusion broth to a concentration of 1:8,000,000 or 1:16,000,000. Varying amounts of this solution were distributed in a series of tubes as indicated in table 2, and the volume was adjusted to 0.8 ml. These volumes were rounded off to the nearest 0.05 ml as indicated in the table. To each tube was then added 0.8 ml of a 1.5 per cent suspension of defibrinated rabbit blood in infusion broth, previously inoculated with 0.0003 volumes of an 18-hour culture (in blood broth) of group A hemolytic streptococcus (strain C-203). The tubes were shaken and the results of hemolysis read after 18 to 24 hours at 37 C. The end point was usually sharply defined, despite the relatively small gradations in concentration; occasionally, however, a tube showing complete inhibition and one showing definite hemolysis would be separated by one tube showing only a barely perceptible trace of hemolysis. In such cases the end point was arbitrarily interpolated midway between the concentrations of penicillin which completely and almost completely inhibited the hemolytic reaction.

TABLE 2

*Method used for assay of streptococcidal activity**

Unknown penicillin solution: ml of 1:16,000,000 dilution in infusion broth	0.8	0.64	0.48	0.4	0.32	0.24	0.2	0.16	0.12	0.1
Broth diluent, ml	0	0.16 (0.15)	0.32 (0.3)	0.4 (0.4)	0.48 (0.5)	0.56 (0.55)	0.6 (0.6)	0.64 (0.65)	0.68 (0.7)	0.7 (0.7)
1.5 per cent defibrinated rabbit blood in beef broth, inoculated with 0.03 volumes of 18-hour culture of group A *S. pyogenes* (C-203)	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Results of hemolysis after 18 hours at 37 C	0 0	0 0	+ +	+ +	+ +	+ +	+	+	+	+
Results in reference standard (1:16,000,000 G)	0 0	0 0	0 0	0 0	+ +	+ +				

Conclusion: Since the solution of G inhibited hemolysis in 0.4 ml, and the unknown only in 0.64, the latter is approximately $\frac{0.4}{0.64}$ = 63 per cent as active as G. Clearly, assays such as the foregoing give only a range of activity. In the specific example, the unknown was more than 50 per cent $\left(\frac{0.32}{0.64}\right)$, and less than 83 per cent $\left(\frac{0.4}{0.48}\right)$, as active as G. Finer interpolation would be necessary to delimit the activity more precisely.

* Subsequent to the completion of the experiments described in the present paper, the method of assay has been modified in the following particulars: (1) finer interpolations have been made in the amounts of the unknown solution: i.e., 0.8, 0.72, 0.6, 0.48, 0.4, etc., instead of the amounts indicated in the table; (2) the concentration of defibrinated rabbit blood in the inoculum has been increased from 1.5 to 4 per cent, and the volume of cell suspension used in the assay decreased from 0.8 to 0.4 ml. A heavily seeded 6- to 8-hour culture is now used instead of an 18-hour culture to inoculate the blood broth (0.04 ml of culture per 100 ml broth). The net result of these changes has been further to increase the sensitivity and accuracy of the assay.

Subsequent to the completion of the experiments described in the present paper, the method has been modified in several particulars as described in a footnote to table 2.

Assay of spirocheticidal action. The stock solution of penicillin was diluted to a concentration of 1:1,000,000 in beef infusion broth. Varying amounts were distributed in a series of tubes as indicated in table 3, and the volumes adjusted to 8 ml with Brewer's thioglycolate medium (Brewer, 1940) containing 0.14 per cent agar in order to promote colony formation, rather than diffuse growth. One ml of rabbit serum previously heated for 2 hours at 63 C was added to all the tubes, which were then inoculated with 1 ml of medium containing 10^3 spirochetes (*Spirochaeta pallida*, Reiter strain). The latter was obtained by appropriate dilution of an actively growing 48-hour culture on thioglycolate medium. The concentration of organisms in the original culture was determined by direct enumeration of the average number per microscopic field (dark field), after the method of Morgan and Vryonis (1938). The thickness of the film was fixed by using a measured drop under a cover slip of known area. Calibration of the area of the microscopic field gave directly the volume of fluid represented by one field, and thus the number per ml.

TABLE 3

Illustrating method used for assays of spirocheticidal activity

Unknown penicillin solution, ml of 1:1,000,000 dilution in beef infusion broth	0.8	0.6	0.4	0.3	0.2	0.15	0.1	0.075	0.05
Ml of thioglycolate medium*	7.2	7.4	7.6	7.7	7.8	7.85	7.9	7.9	7.95
	One ml rabbit serum, previously heated at 63 C for 2 hours, added to all the tubes, followed by 1 ml of thioglycolate medium containing 1,000 organisms (*Spirochaeta pallida*, Reiter) from 48-hour culture								
Number of colonies after 7 days at 37 C	0	0	13	61					
			End point (20 colonies) = 0.36 ml						
Number of colonies in standard (1:1,000,000 dilution of G)	0	0	0	0	29	58			
			End point (20 colonies) = 0.23 ml						

Conclusion: Since the end point (20 colonies) was 0.23 ml of a 1:1,000,000 dilution of G, and 0.36 of a 1:1,000,000 dilution of the unknown, the latter was 64 per cent ($\frac{23}{36}$) as active as G.

* Brewer's thioglycolate medium, containing 0.14 per cent agar to promote colony formation rather than diffuse growth.

The number of colonies in each tube was read after 7 to 10 days' incubation at 37 C. The end point was that amount of penicillin which reduced the total number of colonies to 20, and was obtained by interpolation as indicated in table 3. The reproducibility of these assays is apparent in table 5.

EXPERIMENTAL RESULTS

The results of the individual assays are summarized in tables 4 and 5. Against the C-203 strain of *Streptococcus pyogenes* the activities per mg of two lots of

TABLE 4

The relative streptococcidal activity of crystalline penicillins F, G, K, and X in vitro (gravimetric), referred to that of G as 100†

(Single strain of group A hemolytic streptococcus, C-203, used throughout)

TYPE OF PENICILLIN	LOT NO.	WEIGHED SAMPLE NUMBER				MEAN ACTIVITY OF INDIVIDUAL LOTS	AVERAGE ACTIVITY OF CRYSTALLINE SAMPLES
		1	2	3	4		
F	I	80		100, 100*, 93, 100, 75*	100, 100*, 85	93	82
	II			67, 85, 42, 75*	80, 95	71	
G	I		85*		100, 100	95	100
	II		100			100	
	III		100			100	
K	I			108, 110*, 130, 125, 110*	140	121	120
X	I	100, 100*, 160, 160*	150, 150*	133*, 130, 125, 166*		137	140
	II		160, 160*			160	
	III			100, 120*, 100, 120*, 133		117	

* Activity of solutions prepared aseptically, without Seitz filtration, referred to that of solution of G similarly prepared. All other assays carried out with Seitz-filtered solutions (cf. text).

† Subsequent to the completion of the experiments summarized in table 4, in a second series of 10 assays with a somewhat more refined method (cf. footnote to table 2), the relative activities of penicillins F, G, K, and X averaged 70, 100, 110, and 130, respectively, in reasonably good agreement with the data of table 4.

TABLE 5

The relative spirocheticidal activity in vitro (Spirochaeta pallida, Reiter) of penicillins F, G, K, and X

(Activities per mg are referred to that of G as 100)

TYPE OF PENICILLIN	LOT NO.	WEIGHED SAMPLE NUMBER			MEAN ACTIVITY OF INDIVIDUAL LOTS	AVERAGE ACTIVITY OF CRYSTALLINE SAMPLES
		1	2	3		
F	I			62, 65, 46, 57	58	53
	II			40, 41, 48, 58	47	
G	I	100	100	100	100	100
	II	110	100	100		
	III		100	100		
K	I			51, 79, 94, 78	76	76
X	I	60	50	55, 50, 56	54	51
	II		50		50	
	III			44, 52	48	

crystalline F averaged 93 and 71, referred to that of G as 100; three lots of G assayed identically; a single lot of K had an average activity of 120; and the three lots of X had activities of 137, 160, and 117. The mean gravimetric activity of F, G, K, and X against this particular strain of streptococcus *in vitro* was therefore 82, 100, 120, and 140, respectively.

Subsequent to the completion of the experiments summarized in table 4, in a second series of 10 assays with a somewhat more refined method (cf. footnote to table 2), the relative activities of penicillins F, G, K, and X averaged 70, 100, 110, and 130, respectively, in reasonably good agreement with the data of table 4.

Against *Spirochaeta pallida* (Reiter), the two lots of crystalline F had average activities per mg of 58 and 47, referred to that of G as 100; the three lots of G assayed identically; the single crystalline sample of K averaged an activity of 76; and three different lots of X assayed at 54, 50, and 48. The mean activities per mg of F, G, K, and X against this strain of spirochete *in vitro* were 53, 100, 75, and 50.

TABLE 6

The relative activity in vitro of penicillins F, G, K and X against Staphylococcus aureus, Streptococcus pyogenes (C-203), and Spirochaeta pallida (Reiter)

(All values have been referred to that of G as 100)

	RELATIVE ACTIVITY PER MG PENICILLIN				RELATIVE ACTIVITY PER "UNIT" PENICILLIN			
	*Staphylococcus**	*Streptococcus*		*Spirochaeta pallida*	*Staphylococcus*	*Streptococcus*		*Spirochaeta pallida*
		I	II			I	II	
F	90	82	70	53	100	91	80	60
G	100	100	100	100	100	100	100	100
K	138	120	110	76	100	87	80	55
X	54	140	130	51	100	260	240	95

* Using the values of 1,550, 1,667, 2,300, and 900 units per mg for F, G, K, and X, respectively.

For comparison, the stated values for the activities of these penicillins against *Staphylococcus aureus* are 1,550, 1,667, 2,300, and 900 units per mg, or relative activities of 90, 100, 140, and 55, respectively.

In the right-hand section of table 6 the foregoing gravimetric assays have been recalculated to show the relative activities of the four penicillins per staphylococcidal "unit" rather than per mg. So expressed, the relative activities of F, G, K, and X against the streptococcus become 90, 100, 87, and 260, respectively; and their relative activities against the Reiter spirochete become 60, 100, 55, and 95.

DISCUSSION

Penicillins F, G, K, and X differ significantly in their direct bactericidal activity per mg (Veldee *et al.*, 1945). In their staphylococcidal activity there is a 2.5-fold difference between the least and the most active against *Staphylococcus* *aureus* (X with 900 units per mg and K with 2,300 units per mg). The present data indicate differences of the same order of magnitude in their activity against either streptococci or spirochetes, i.e., a 1.8-fold difference between the least active (F) and most active (X) against the C-203 strain of group A hemolytic streptococcus, and a 2-fold difference between F and G in the case of cultures *Spirochaeta pallida* (Reiter). The relative activity of the penicillins was not regular but varied unpredictably from organism to organism. With *Staphylococcus aureus*, activity increased in the order $X < F < G < K$; the order was $F < G < K < X$ with the present strain of streptococcus, and $X = F < K < G$ in the case of the spirochete.

Maximum differences in bactericidal activity of approximately twofold would not present a serious therapeutic problem, particularly since commercial penicillins, containing these four fractions in varying proportion, would differ by something less than twofold in their over-all activity against a given organism. It is, however, conceivable that the same differences in the chemical configuration on the side group that determine the varying bactericidal activities of these penicillins may also variably affect the rate of their excretion, diffusion, or inactivation *in vitro*. Under such circumstances, their therapeutic activity may vary to a much greater extent than their direct bactericidal action *in vitro*, and not necessarily in parallel to the latter. Studies in this direction are now in progress (Eagle and Musselman, 1946).

SUMMARY

Two lots of crystalline penicillin F, three lots of G, one of K, and three of X have been assayed for streptococcidal activity (group A hemolytic streptococcus, C-203) and spirocheticidal activity (*Spirochaeta pallida*, Reiter strain) *in vitro*.

The relative activities per mg of these crystalline preparations against the streptococcus averaged 82, 100, 120, and 140 for F, G, K, and X, respectively, in one series of assays, and 70, 100, 110, and 130 in a second series. The corresponding activities against the spirochete were 53, 100, 76, and 51, respectively. In comparison, the relative activity of these four penicillins against *Staphylococcus aureus* are stated to be 90, 100, 138, and 54.

Per (staphylococcidal) "unit," the activities of F, G, K, and X against the streptococcus averaged 85, 100, 85, and 250, and against the spirochete, 60, 100, 55, and 95.

With each of the three organisms there was therefore approximately a twofold difference between the least active and most active penicillin; but their relative activity varied unpredictably from organism to organism. The order of increasing activity was $X < F < G < K$ for *Staphylococcus aureus*, $F < G < K < X$ for the streptococcus here studied, and $X = F < K < G$ for the spirochete.

The therapeutic significance of these data is discussed in the text.

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THE ACTIVITY OF PENICILLIN IN RELATION TO BACTERIAL SPORES AND THE PRESERVATION OF MILK

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The activity of penicillin against the spores of *Bacillus subtilis*, *Bacillus megatherium*, *Bacillus cereus*, and *Bacillus stearothermophilus*, was reported recently (Curran and Evans, 1945*a*). As little as 5 Oxford units per ml of the drug effected a marked reduction in the number of viable spores in milk in three of the four species when the samples were suitably incubated for periods of 5 and 27 hours. The plate method of enumeration was employed. Later, Gardner (1945) reported a similar observation based upon the direct microscopical examination of the spores of *Bacillus subtilis* and *Bacillus anthracis* (avirulent) cultivated in the presence of penicillin on nutrient agar blocks.

These findings indicated certain possibilities in the application of penicillin as a preserving agent. Whether, with opportunity for prolonged action, penicillin would be able ultimately to kill all spores of a susceptible species was a question not yet answered, nor was it known whether spores of penicillin-resistant species could be controlled by relatively high concentrations of penicillin. More extended information as to the relative proportion and distribution of susceptible and refractory species was also essential to a critical evaluation of penicillin as a preservative.

In this report an attempt has been made to answer some of these questions. The observations have been limited to the action of penicillin in milk.

METHODS AND MATERIALS

The organisms and their sources were *Bacillus subtilis*, strains 6, 15U, 4149, and LB, recovered from spoiled commercially processed evaporated milk either by the American Can Company or by this laboratory; *Bacillus metiens* and *Bacillus subtilis*, Merck and FDA, from Dr. W. A. Randall of the Food and Drug Administration; *Bacillus albolactis* from the Bureau of Dairy Industry culture collection; the anaerobes 3679, a nontoxigenic proteolytic culture, and *Clostridium botulinum* (62A) from Mr. John Yesair, National Canners Association; and the remaining cultures from the N. R. Smith collection through the courtesy of Dr. Ruth Gordon.

The spores of the aerobic species[1] were produced on standard beef extract tryptone agar medium incubated at the optimum temperature of the organism. They were collected and prepared for use as described by Curran and Evans (1945*b*). Aqueous suspensions of the washed spores were inoculated directly into the test sample. The spores of anaerobes were produced in casein digest

[1] *B. subtilis* FDA was cultivated on glucose agar to prevent its dissociation.

medium, well-mixed suspensions of which served directly as inocula. Pour plates were used for count determinations. For the aerobes the plating medium was that used for the production of spores with 0.5 per cent glucose added. For the anaerobes Brewer plates (Brewer, 1942) and B-B-L anaerobic agar were used. Plates were incubated at the optimum temperature of the organism and counted after 2 to 3 days. Heat treatments were conducted in a thermostatically controlled glycerol bath (±0.5 C).

Some degree of pretreatment heating of milk samples is necessary if the preserving action of penicillin is to be efficiently utilized. This kills many spores, all nonsporulating species which may be resistant to penicillin, and also the vegetative forms of sporing species. Some of the latter are less inhibited by penicillin than are the spores from which they are derived. Mild heating serves also to inactivate preformed microbial penicillinase.

Fresh raw milk and sterile (autoclaved) skim milk, tubed in 10-ml quantities, were the culture mediums. Whenever anaerobic cultures were employed, the dissolved oxygen was driven off by mild heat before inoculation and the samples were sealed by means of sterile agar and mineral oil.

Penicillin[2] sodium was used throughout. The solvent was a buffer mixture consisting of 1 per cent each of K_2HPO_4 and KH_2PO_4, pH 6.0. The penicillinase[3] was the commercial product, dissolved in sterile distilled water.

For testing the presence of botulinus toxin 0.1-ml quantities of each incubated test culture were carefully introduced directly into the stomachs of each of two mice by means of a blunted hypodermic needle. Similar quantities of each incubated or unincubated control were similarly fed to two mice. Animals showing any evidence of bleeding immediately after the operation were discarded and replacements provided. Mice fed from the same culture were placed together in separate cages and observed daily for a period of 5 days. The mice were 3 to 4 months old and weighed about 30 g each.

The activity of penicillin was tested against a group of 15 aerobic and 2 anaerobic strains representing species which are disseminated widely in nature. The washed spores were uniformly dispersed in sterile (autoclaved) milk in cell concentrations ranging from 50 to 100,000 per ml, heated at 95 C for 15 minutes,[4] and cooled; penicillin was added; and the samples were stored at 30 C. Spoilage was a measure of the capacity of the spores to germinate and to vegetate within the recorded period of observation.

RESULTS

Some measure of inhibition or destruction of the spores was found in all except 4 closely related species. The latter, *Bacillus cereus* (7 strains), *Bacillus mycoides* (2 strains), *Bacillus metiens*, and *Bacillus albolactis*, induced spoilage both with and without penicillin within 1 week. Samples containing penicillin which spoiled in 4 weeks were *Bacillus subtilis* Ford, *Bacillus subtilis* var. *aterrimus* 230, *Bacillus subtilis-niger* 6454, and *Bacillus alvei* 680; in 8 weeks, *Bacillus subtilis* Marburg and *Bacillus subtilis* from Merck; in 12 weeks, *Bacillus alvei* 395, 685, and 686; and in 16 weeks, *Bacillus subtilis* 4149, 3679 (anaerobe), and *Clostridium botulinum* 62A. The following members of the genus *Bacillus* produced no change in the presence of penicillin during 16 weeks of observation: *B. subtilis* 6598, 6, 15U, LB, FDA; *B. pumilus* 7061; *B. brevis* 8185, 8186, Penn. S; *B. alvei* 683, 684, 750, 811; *B. firmus* 8247; *B. laterosporus* 8248, 9141; *B. megatherium* 234, 389, 696, 753, 931; *B. circulans* 7049; *B. polymyxa* 8240; *B. sphaericus* 7054, 4525; *B. macerans* 7069. In the absence of penicillin, all except 5 samples spoiled the milk in from 1 to 2 weeks. *B. sphaericus* (2), *B. circulans*, *B. firmus*, and certain strains of *B. alvei* required from 3 to 4 weeks to produce visible spoilage.

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[2] Kindly supplied by Chas. Pfizer and Co., Brooklyn, New York.

[3] Obtained through the courtesy of the Schenley Research Institute, Lawrenceburg, Indiana.

[4] Primarily to activate the spores and to kill nonsporulating organisms.

These samples, with few exceptions, did not spoil when they were treated with sterile penicillinase at the end of the incubation period and were then incubated, thus indicating the absence of viable organisms. The results with *Bacillus subtilis* differed with the strain, from delayed spoilage to sterilization of the sample. The data on *B. cereus*, *B. megatherium*, and *B. subtilis* are in accord with our previous findings (Curran and Evans, 1945*a*). In the samples containing anaerobes, penicillin delayed but did not prevent spoilage.

Four strains of *B. subtilis* (FDA, 15U, 4149, and LB), *B. brevis* (Penn S), and *B. circulans* (7049) evidenced heat activation (Curran and Evans, 1945*b*). In all of these spoilage was either greatly delayed or prevented.

Although the correlation is not complete, resistance to penicillin among species and among the strains of a species is usually associated with thermolability; pronounced susceptibility to penicillin on the other hand occurs usually among the species of moderate and high thermal resistance. Within a given species, however, the reverse seems to be true; viz., the heat-resistant (surviving 95 C for 15 minutes) spores comprise the largest proportion of penicillin-resistant spores.

Action of penicillin against species relatively resistant to the drug. Some further observations were made upon the action of penicillin against penicillin-resistant species. Four cultures, *Bacillus cereus* 369 and 401, *Bacillus mycoides* 6462, and *Bacillus metiens* were studied. Spores of these cultures were seeded into raw milk in varying concentrations; preliminary heating at 95 C for 15 minutes was combined with incubation of the samples at 30 C for 5 hours, followed by heating at 85 C for 15 minutes. The results (not detailed) revealed that only those samples containing less than 23 viable spores per ml after the initial heating were preserved. These results suggest that all species produce spores which are susceptible to penicillin in some degree—the species differing in the relative proportion of susceptible and resistant cells.

Action of penicillin against naturally produced spores. It is generally recognized that organisms developing in natural environments are often endowed with greater resistance than those produced in the laboratory. To test the action of penicillin (5 u per ml) against naturally produced spores, raw milk was treated

TABLE 1

The preserving action of penicillin (5 u per ml) in milk not artificially inoculated

DATE	TOTAL COUNT (AEROBIC)	COUNT AFTER HEATING 95 C—15 MIN (AEROBIC)	PRELIMINARY INCUBATION	AFTER 3 MONTHS' STORAGE AT 30 C	
				Aerobic	Anaerobic
	per ml	*per ml*			
21/3	16,000	2.6	None	U	
23/3	1,500	1.3	None	U	
28/3	10,400	2.3	None	U	
4/4	68,000	134 (147)*	None	S	
11/4	44,600	39	None	S	
18/4	77,000	15	None	S	
25/4	17,700	1	None	U	
3/5	2,600	4	None	U	
9/5	2,100	2	5 hr at 30 C plus 85 C for 15 min	U	
16/5		3	5 hr at 30 C plus 85 C for 15 min	U	
25/6	4,300	7 (8)	5 hr at 30 C plus 85 C for 15 min	U	
10/7	28,000	5 (11)	5 hr at 30 C plus 85 C for 15 min	U	
14/8	27,000	12 (58)	5 hr at 30 C plus 85 C for 15 min	U	
21/8	116,000	10 (17)	5 hr at 30 C plus 85 C for 15 min	U	
23/8	16,000	6 (13)	5 hr at 30 C plus 85 C for 15 min	U	U
28/8	51,000	6 (19)	5 hr at 30 C plus 85 C for 15 min	U	
5/9	24,000	19 (35)	5 hr at 30 C plus 85 C for 15 min	S	S
7/9	133,000	2 (35)	5 hr at 30 C plus 85 C for 15 min	U	S
12/9	169,000	3	5 hr at 30 C plus 85 C for 15 min	S	
20/9	190,000	16 (70)	5 hr at 30 C plus 85 C for 15 min	S	S
21/9	14,000	1 (27)	5 hr at 30 C plus 85 C for 15 min	U	U
25/9	54,000	46	5 hr at 30 C plus 85 C for 15 min	S	S
26/9	98,000	24	5 hr at 30 C plus 85 C for 15 min	U	U
28/9	6,000	1	5 hr at 30 C plus 85 C for 15 min	U	S
6/11	25,000	4 (13)	5 hr at 30 C plus 85 C for 15 min	U	U

Cereus-mycoides types predominated in the organisms which survived preliminary heating.

U = Unchanged.

S = Spoiled.

* Figures in parentheses = after heating at 85 C for 15 min.

after being heated at 95 C for 15 minutes, with and without a short incubation period. Anaerobic, as well as aerobic, cultivation was employed in part of these tests. The milk was collected over a period of 8 months to provide seasonal variations in the spore flora.

The results (table 1) show that the count of the aerobic thermostable spores was low throughout. Qualitatively, *B. cereus* and *B. mycoides* types greatly predominated. In only 4 of the 25 samples did the count of thermostable (aerobic) spores exceed 20 per ml. There is little correlation between the total count and the number of spores which survived heating at 95 C for 15 minutes; also there is little correlation between the total count (vegetative and spores)

TABLE 2

The sterilizing levels of penicillin in milk containing both naturally occurring and artificially introduced penicillin-resistant spores (Bacillus cereus 720)

VIABLE SPORES AFTER INOCULATION	PRELIMINARY TREATMENT	PENICILLIN	CONDITION OF MILK AFTER 3 MONTHS' STORAGE AT 30 C		
			B. cereus	Penicillin	*B. mycoides*
per ml		*u/ml*		*u/ml*	
220,000	95 C-15 min; no incub.	100	U	100	S 1 wk
220,000	95 C-15 min; no incub.	500	U	500	U
220,000	95 C-15 min; 30 C-5 hr; 85 C-15 min	50	S 1 wk	100	S 1 wk
220,000	95 C-15 min; 30 C-5 hr; 85 C-15 min	100	U	500	U
22,000	95 C-15 min; no incub.	50	S 8 wk	50	S 1 wk
22,000	95 C-15 min; no incub.	100	S 8 wk	100	U
22,000	95 C-15 min; 30 C-5 hr; 85 C-15 min	10	S 2 wk	10	S 1 wk
22,000	95 C-15 min; 30 C-5 hr; 85 C-15 min	50	S 8 wk	50	U
2,200	95 C-15 min; no incub.	50	S 2 wk	50	U
2,200	95 C-15 min; no incub.	100	S 8 wk	100	U
2,200	95 C-15 min; 30 C-5 hr; 85 C-15 min	10	S 8 wk	10	S 4 wk
2,200	95 C-15 min; 30 C-5 hr; 85 C-15 min	50	U	50	U
200	95 C-15 min; no incub.	50	S 2 wk	50	U
200	95 C-15 min; no incub.	100	S 8 wk	100	U
200	95 C-15 min; 30 C-5 hr; 85 C-15 min	10	S 1 wk	10	U
200	95 C-15 min; 30 C-5 hr; 85 C-15 min	50	U	50	U
No inoc.	No heat; no incub.	500 aerobic	S 1 wk		
		500 anaerobic	S 1 wk		
No inoc.	No heat; no incub.	1,000 aerobic	S 1 wk		
		1,000 anaerobic	S 2 wk		
No inoc.	95 C-15 min; no incub.	50 aerobic	S 2 wk		
		50 anaerobic	S		
No inoc.	95 C-15 min; no incub.	100 aerobic	S 8 wk		
		100 anaerobic	S 8 wk		
No inoc.	95 C-15 min; 30 C-5 hr; 85 C-15 min	10 aerobic	S 1 wk		
		10 anaerobic	S 1 wk		
No inoc.	95 C-15 min; 30 C-5 hr; 85 C-15 min	50 aerobic	S 4 wk		
		50 anaerobic	S 1 wk		

Raw milk, unheated, 48,000 per ml; raw milk after 95 C for 15 min, 48 per ml.
Aerobic and anaerobic refer to storage conditions.

and spoilage, but a rather close correlation between the thermostable spore count and spoilage. With one exception, in all the samples that spoiled the spore count was 15 to 134 per ml, whereas, with one exception, all the preserved samples initially contained 10 or less thermostable spores per ml. As would be expected, the counts after heating at 85 C for 15 minutes were substantially higher than those after heating at 95 C for 15 minutes; however, the average ratio of difference was in this instance much smaller than that found in previous experiments in which artificially produced spores were used. This indicates that the naturally occurring *B. cereus* and *B. mycoides* spores are relatively more resistant to heat than are the artificially produced spores of these species.

TABLE 3

*The effect of penicillin upon the preservation of autoclaved milk seeded with the spores of a penicillin-sensitive species**

NO. OF SPORES AFTER INOCULATION	PRELIMINARY TREATMENT	LEVEL OF PENICILLIN	CONDITION OF MILK AFTER STORAGE AT 30 C FOR			
			10 days	1 month	2 months	3 months
per ml		*u/ml*				
119,000	95 C-15 min	5	10-U	3-S 7-U	8-S 2-U	10-S
119,000	95 C-15 min	50	2-U	2-U	2-U	1-S 1-U
11,900	95 C-15 min	5	10-U	10-U	10-U	10-U
11,900	95 C-15 min	50	2-U	2-U	2-U	2-U
1,190	95 C-15 min	5	10-U	10-U	10-U	10-U
1,190	95 C-15 min	50	2-U	2-U	2-U	2-U
710	No heat	5	7-S 3-U	9-S 1-U	9-S 1-U	9-S 1-U
710	No heat	20	2-U	2-U	2-U	1-S 1-U
710	95 C-15 min	5	10-U	10-U	10-U	10-U
710	95 C-15 min	20	2-U	2-U	2-U	2-U
65	No heat	5	1-S 9-U	1-S 9-U	1-S 9-U	1-S 9-U
65	No heat	20	2-U	2-U	2-U	2-U
65	95 C-15 min	5	10-U	10-U	10-U	10-U
65	95 C-15 min	20	2-U	2-U	2-U	2-U
65	95 C-15 min	No penicillin	2-S			

Numbers in storage period columns refer to the number of tubes.

* *Bacillus subtilis* var. *aterrimus*.

The ineffectiveness of penicillin against spores capable of growing anaerobically is apparent from these data. All samples that spoiled under anaerobic cultivation included all those that spoiled aerobically and some that did not.

Levels of penicillin required for sterilization. In table 2 are shown the sterilizing levels of penicillin in milk containing both naturally occurring and artificially introduced penicillin-resistant spores. From 100 to 500 u per ml of penicillin were required to preserve the inoculated cultures depending upon the initial concentration of spores and the prestorage treatment. The preliminary incubation reduced the amount of penicillin required for preservation in some samples; in others no differences are apparent. The effect of concentration of spores upon the limiting concentration of penicillin varied greatly with the two species. The data on the uninoculated sample furnish further evidence of the presence in milk of spores resistant to penicillin and of the uncertainty of penicillin as an aid to preservation. As is evident in table 2, in the absence of pretreatment heating the preservation of milk by penicillin is practically unattainable. As much as 1,000 u per ml in this instance was ineffective in preventing rapid spoilage.

TABLE 4

The influence of penicillin upon the development of the spores of Clostridium botulinum in milk and upon the formation of toxin

VIABLE SPORES AFTER INOCULATION	TREATMENT	CONDITION OF CULTURE AFTER 7 WEEKS, INCUBATION	CULTURE AFTER 13 WEEKS' INCUBATION	
			Condition	Toxicity* (Mice)
per ml				
80	No penicillin	GP—3 weeks	GP	1 dead <24 hr 1 very sick
80	Penicillin 5 u/ml	U	U	2 normal
800	No penicillin	GP—3 weeks	GP	1 dead <24 hr 1 dead <48 hr
800	Penicillin 5 u/ml	U	U	2 normal
8,000	No penicillin	GP—3 weeks	GP	1 dead <24 hr 1 very sick
8,000	Penicillin 5 u/ml	U	U	2 normal
80,000	No penicillin	GP—3 weeks	GP	2 dead <48 hr
80,000	Penicillin 5 u/ml	GP—(6 weeks)	GP	2 dead <48 hr
80,000	Penicillin 50 u/ml	U	U	
800,000	No penicillin	GP—10 days	GP	2 dead <36 hr
800,000	Penicillin 50 u/ml	GP—3 weeks	GP	1 dead 48 hr 1 very sick
800,000	Penicillin 100 u/ml	GP—3 weeks	GP	
No inoc.	Penicillin 5 u/ml	U	U	2 normal
No inoc.	No penicillin	U	U	2 normal
318,000	Initial inoculum	U	U	2 normal

GP = Gas, peptonization.
U = Unchanged.

*0.1 ml of the incubated test culture was forcibly fed to each mouse. All the sick animals evidenced the typical symptoms of botulinus intoxication.

Action of penicillin against a species relatively sensitive to the drug. In the next experiment, spores of a penicillin-sensitive species, *Bacillus subtilis* var. *aterrimus*, served as inocula in sterile milk. This strain, together with *Bacillus niger* and the Ford strain of *B. subtilis*, is more resistant to penicillin than other members of the subtilis group. The data (table 3) show that the number of spores has an important influence upon the treatment necessary to ensure sterilization. When the number of spores was relatively high, 5 u per ml of penicillin was not a consistently effective preservative even when combined with preliminary heating. When the spores numbered 10,000 per ml or less, 5 u per ml was effective when used in conjunction with prior heating. Without the latter, sterility was not attained with as few as 65 spores per ml, although 20 u per ml did preserve these samples.

The action of penicillin against the spores of Clostridium botulinum. Varying numbers of spores of *Clostridium botulinum* were incubated anaerobically in milk with different concentrations of penicillin. At the recorded intervals, gross changes in the appearance of the samples were noted and animal feeding tests[5] performed. The data presented in table 4 show that penicillin in low concentration (5 u per ml) greatly delayed growth and the formation of toxin when the concentration of spores was substantially below 800,000 per ml. At the latter level, the effect of penicillin up to 100 u per ml was almost negligible; however, in the presence of relatively few spores (< 8,000 per ml) the growth- and toxin-delaying action of 5 u per ml of penicillin was pronounced, the data giving no indication as to the limits of the sporistatic period. No explanation is offered for the lack of complete uniformity in some of the feeding tests. The importance of the number of spores in determining the length of the inactive period suggests that a very small proportion of the spores in the initial population was insusceptible to the inhibitory action of penicillin, rapid spoilage in a particular instance depending upon the presence in the sample of 1 or more resistant cells. Bigger (1944) has applied the name "persisters" to the very small minority of *Staphylococcus pyogenes* cells found to be resistant to the activity of penicillin. The resistant cells rarely exceeded 1 per million of the cocci originally present. A closely analogous situation seems to exist for spores.

DISCUSSION

It is evident from the foregoing report that penicillin in low concentration (5 u per ml) is remarkably sporistatic and sporicidal for a wide range of organisms. Certain deficiencies of the drug as a spore-controlling agent are also apparent; a small but widely disseminated group of sporing aerobes is strongly resistant to penicillin. When such spores are present, even in small numbers, penicillin is not an effective preserving agent except in concentrations which would be impracticable. With certain other aerobes, penicillin in low concentration delays but does not consistently prevent the germination of their spores and subsequent growth. Data obtained with anaerobic sporeformers show that the spores of some species can develop rather quickly in the presence of about 5 u per ml of penicillin. Likewise, this level of penicillin will not consistently prevent the growth and the formation of toxin originating from the spores of *Clostridium bolulinum*, though these may be greatly delayed.

Delayed spoilage indicates an inhibiting action upon the germination of the spores. This continues presumably until the penicillin falls below a sporistatic level; in some strains of *B. subtilis* the threshold concentration is less than 0.015 u per ml. In view of the rather slow deterioration of penicillin even at incubator temperatures (Rammelkamp and Helm, 1943; Kirby, 1944; Benedict *et al.*, 1945), 5 u per ml of penicillin might be expected to exert a prolonged sporistatic action against susceptible species.

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[5] Our thanks are due to Dr. Samuel R. Hall of this Bureau for his assistance in the feeding and care of the animals.

The possible utility of penicillin in the preservation of nonfood materials would depend upon the reaction of such materials and upon the kind and number of spores encountered. In combination with mild heating penicillin might find application in special situations, either as a preservative or spoilage-delaying agent.

SUMMARY

A study was made of the preserving action of penicillin in milk containing viable bacterial spores. Fifteen aerobic and two anaerobic species were examined.

In their reaction to penicillin, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus albolactis*, and *Bacillus metiens* were relatively resistant. When they were present penicillin was not an effective preserving agent except in concentrations that would be impracticable. The remaining 13 species were relatively susceptible to penicillin as was manifested by strong sporicidal or prolonged sporistatic activity in a drug concentration of 5 u per ml. This concentration sterilized many of these cultures.

Five units per ml of penicillin greatly delayed but did not prevent spoilage by *Clostridium botulinum* and an unidentified anaerobic species (3679). In the former, toxin formation accompanied spoilage, both in the control and penicillin samples. The sporistatic period varied with the concentration of spores and the level of penicillin. The data indicate that an extremely small proportion of the spores of *Clostridium botulinum* are highly resistant to the sporistatic action of penicillin.

The evidence suggests that all spore cultures contain spores susceptible to penicillin, the species differing in the relative proportion of resistant and sensitive cells.

On the basis of this study, it is concluded that penicillin has no application in the preservation of food. In combination with mild heating, it might have utility as a preservative or spoilage-delaying agent in certain nonfood materials.

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THE NATURE OF THE ACID-FAST STAIN

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A satisfactory explanation of the nature of the Ziehl-Neelsen stain remains to be formulated. It is agreed that disruption of the cell membrane by chemical or physical means is accompanied by a loss of acid-fastness (Long, 1922–23; Yegian and Porter, 1944), and that acid-fast lipids peculiar to the mycobacteria exist (Tamura, 1913; Anderson, 1929). A critical review of the literature on the subject has been presented by Wells and Long (1932). Since then a new and major observation has been made by Yegian and Baisden (1942) and Porter and Yegian (1945), who have demonstrated that beading and Much's granules are artifacts dependent upon the staining procedure. No theory of the acid-fast stain offered to date can satisfactorily explain the origin of these artifacts. The present paper develops a hypothesis which, if it can stand the test of time, presumably can account for the properties of acid-fast cells when stained with carbol-fuchsin and for the artifacts that have been observed.

Carbol-fuchsin is a mixture including phenol, water, and a dye (basic fuchsin) much less soluble in water than in phenol. When carbol-fuchsin penetrates into a cell, the distribution of phenol and dye within the cell should be a function of their concentration in the reagent and their *solubility* in the lipids and water phases present within the cell, assuming the absence of chemical reactions. Factors which influence the mutual solubility of the phenol and dye, or phenol, dye, and lipids, should affect the appearance of the stained cell.

When an aqueous solution of phenol is added to a lipid (water-insoluble) two phases will result, a water and a lipid phase. The phenol will be distributed among the phases depending upon its relative solubility in the phases. For example, with oleic acid the phenol would tend to concentrate in the oleic acid phase. Basic fuchsin and a great variety of other dyes are more soluble in phenol and some other organic solvents than in water. Thus when oleic acid is added to carbol-fuchsin and vigorously mixed, and then centrifuged, two phases separate out, a top layer of oleic acid and a more lightly colored bottom water layer. The dye and phenol tend to be concentrated in the oleic acid. If a solvent is added, such as ethyl alcohol, in which both water and oleic acid are infinitely soluble, the two phases disappear and a homogeneous colored solution results.

A simple analysis of the solubility of phenol in water as influenced by the presence of other substances, such as basic fuchsin, inorganic salts, and ethyl alcohol, which all occur in carbol-fuchsin, is provided by the determination of the consolute or critical temperature in the presence of these substances. Some of such data are recorded in table 1. As indicated by the rise of the consolute temperature, these data prove that sodium chloride decreases the solubility of phenol in water. The effect of basic fuchsin is more complex. A small amount causes a lowering of the consolute temperature, whereas larger quantities cause a rise. The exact concentration of dye at which this reversal takes place varies for each batch of dye as prepared for commercial use. By adding sufficient dye the solubility of phenol in water can be decreased to less than is normal for a mixture of phenol and water alone. The addition of NaCl, which is soluble in water and not in phenol, accentuates this loss of solubility by phenol. It is also possible to "salt out" the dye in the water phase by adding high enough concentrations of sodium chloride. The end effect on the solubility of phenol in carbol fuchsin would seem to depend on the relative concentrations of inorganic salts, alcohol, and dye, and the exact composition of the batch of basic fuchsin utilized.

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[1] The work reported was initiated at the Louisiana State University School of Medicine, Department of Pathology and Bacteriology, New Orleans, Louisiana, and continued at Camp Detrick.

A generalization which can be made from the work of Yegian and Baisden (1942), Yegian and Budd (1943), and Porter and Yegian (1945) is that *factors which decrease the solubility of phenol in water are those leading to the occurrence of artifacts in the Ziehl-Neelsen procedure.* Thus these investigators add sodium chloride to carbol-fuchsin to get staining solutions which will regularly yield a large percentage of stained "beaded" cells. They have noted that reduction of the concentration of the stain is accompanied by a decrease in beading. Yegian and Budd (1943) have found that carbol-fuchsin made up with the acetate salts of rosaniline and *para*-rosaniline gives more beading than when the chloride dye salts are used. It is significant that the acetate salts are more soluble in alcohol than the chloride salts. Therefore, when equal quantities of saturated alcoholic solutions of these dye salts are used to make up the carbol-fuchsin, it is more likely that with the acetate dye salts high enough concentrations of dye will be present to cause a loss in solubility of the phenol than in the case of the chloride. In agreement with this statement is the reported observation (Yegian and Baisden, 1942) that employment of the acetate dye salts to make up carbol-fuchsin gave a colloidal suspension instead of a clear solution as in the case when the chloride dye salts were used.

The colloidal suspension that is noted for some batches of carbol-fuchsin and for all batches to which inorganic electrolytes are added is the result of the separation of some of the phenol present into a separate phase. When such a solution is centrifuged, two layers are isolated. The more intensely colored layer is at the bottom and consists in part of a viscous liquid with a boiling range of 180 to 185 C. This is within the range of boiling for phenol.

In accordance with the work of Yegian and Baisden (1942) and Porter and Yegian (1945) it has been confirmed that beading occurs in the step of the Ziehl-Neelsen procedure in which the smears are washed with water. The action is sudden and independent of the species of acid-fast organism being studied. But it is dependent on the employment of a suitable batch of carbol-fuchsin and regularly occurs when sodium chloride has been added to the staining solution.

Upon the basis of the foregoing discussion, the beads or artifacts are postulated to be the separation of phenol into a separate liquid phase upon the penetration of water into the cell. In this wise the instantaneous formation, the more or less spherical shape, the concentration of color, and the solubility in alcohol and phenol of the beads can be accounted for. This explanation presumes that the use of carbol-fuchsin in which phenol is present to the extent of saturation or supersaturation with respect to water permits the accumulation of the phenol in a similar state in the bacterial cell. The presence of water-insoluble cellular lipids in which phenol is soluble, or of salts insoluble in phenol, would accentuate the loss of solubility of phenol in water. The role of basic fuchsin is not specific, since malachite green has been substituted for the basic fuchsin in the preparation of staining solutions that favor beading. This proposed explanation of beading is further supported by evidence that the phenomenon is not limited to acid-fast organisms. Four unlike non-acid-fast bacterial species have been studied: *Bacillus megatherium*, *Corynebacterium diptheriae*, *Corynebacterium hofmanni*, and an unidentified species of *Vibrio*. Cells of these species when stained with carbol-fuchsin are rapidly decolorized by alcohol. But when washed with cold water they are decolorized more slowly and at varying rates. Most important of all is the fact that, with the exception of *Bacillus megatherium*, decolorization

TABLE 1

Consolute temperature of phenol-water in the presence of components of carbol-fuchsin

ITEMS ADDED	CONSOLUTE TEMPERATURE (C)
None (control: phenol-water)	68
Ethyl alcohol, 10 per cent	52
NaCl, 0.5 per cent	76
Ethyl alcohol, 10 per cent, and NaCl, 0.5 per cent	58
Coleman and Bell Co.:	
Basic fuchsin, CF-25, 0.5 per cent	54
Basic fuchsin 390547, 0.5 per cent	56
Basic fuchsin 390547, 0.5 per cent and NaCl, 0.5 per cent	77
Allied Chemical and Dye Corp.:	
Para-rosaniline base, 9753, 0.5 per cent	82
Basic fuchsin, NF 40, 0.5 per cent	60
Basic fuchsin, NF 45, 0.5 per cent	61
Basic fuchsin, NF 43, 0.5 per cent	63
Basic fuchsin, NF 43, 1.0 per cent	63
Basic fuchsin, NF 43, 1.0 per cent, and NaCl, 0.5 per cent	78
Basic fuchsin, NF 43, 2.0 per cent	74
Basic fuchsin, NF 43, 2.0 per cent, and NaCl, 0.5 per cent	84
Difco bacto basic fuchsin, 370921	
0.5 per cent	53
0.5 per cent, and NaCl, 0.5 per cent	78
2.0 per cent	42
2.0 per cent, and NaCl, 0.5 per cent	79
6.0 per cent	52
6.0 per cent, and NaCl, 0.5 per cent	88–89

with water is accompanied by the instantaneous appearance of highly colored "beads" when a batch of carbol fuchsin is used to which sodium chloride has been added. Figure 1, nos. 1, 2, and 3 illustrate the situation that obtains with the vibrio. With *Corynebacterium diphtheriae* decolorization with water was least rapid, and the beads were evident for the greatest length of time before being completely washed out of the cells.

If the properties of acid-fast cells stained with carbol-fuchsin are dependent on the relative state of solubility of the phenol in water and cell constituents, it should be possible to decolorize these stained cells merely by immersing them in water at the consolute temperature. The presence of cell constituents in the acid-fast cells that decrease the solubility of phenol in water would not abolish the consolute temperature; they would only raise it. Yegian and Baisden (1942) report that at 90 C *Mycobacterium tuberculosis* (strain H37) can be decolorized with water. This observation has been confirmed. A strain of *Mycobacterium phlei* available in our laboratory can be induced to lose the stain when heated in water at 80 C for several minutes. A strain of the avian tubercle organism was decolorized in water at 70 to 75 C. On the other hand, heating at the same temperature and time with 95 per cent ethyl alcohol does not result in a loss of color. The alcohol-fastness is explained if lipids are present in which a mixture of alcohol and phenol is soluble, and if the lipids do not dissolve out of the cell by the method used for staining when the membrane is intact. Thus the role of the membrane in maintaining the acid-fast properties of cells is harmonious with the suggested thesis. On the other hand, if the cytoplasmic membrane is the principal substrate of the acid-fast property as claimed by

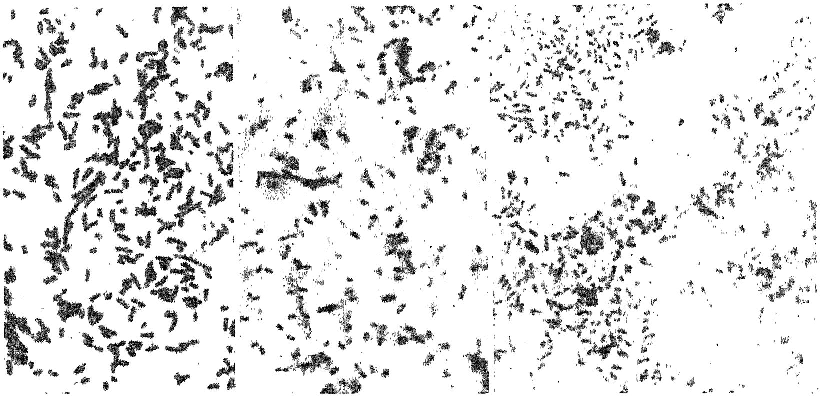
No. 1 No. 2 No. 3

FIG. 1. BEADING OF VIBRIO SP. WHEN STAINED BY ZIEHL-NEELSEN'S CARBOL-FUCHSIN, WITH NaCl ADDED, AND WASHED WITH COLD WATER

No. 1. (Left) Cells stained solidly with carbol-fuchsin. Stain applied and drained off without washing with water. × 1,040

No. 2. (Center) Stained cells washed for a few seconds. Cells lose stain and beading becomes evident. × 1,040

No. 3. (Right) Continued washing in water results in practically all cells becoming beaded. Washing beyond this stage would result in complete loss of stain. × 1,040.

Knaysi (1946), the hypothesis offered would still be applicable. In this case the mutual solubility of the dye solution and lipids of the cytoplasmic membrane would be chiefly concerned.

Added significance of the explanation offered for the beading phenomenon lies in the indication that differences of solubility of phenol in cell constituents (chiefly lipid) may account for the various types of acid-, alkaline-, and alcohol-fastness recognized by the bacteriologist. Since the qualitative and quantitative chemical constitution of different species are dissimilar, the situation within the cell would be complex, and different for each kind of cell. The response to the presence of decolorizing agents would be the summation of all the factors influencing the solubility of phenol and dye within the bacterial cell. The essential difference between the acid-fast and non-acid-fast cell would rest on the greater solubility of the phenol and dye in cell constitutents of acid-fast cells than in the decolorizing solution bathing the cell. In the case of non-acid-fast organisms, the phenol and dye, being more soluble in the decolorizing agent than in the cell constituents, would leave the cells in the presence of the decolorizing agent.

An *in vitro* test of the hypothesis suggested would require a phase rule study of the solubility of carbol-fuchsin and its constituents in cell lipids, and other cellular material, and in combinations of these materials in the proportions they occur in cells.

SUMMARY

The beading of cells when stained with Ziehl-Neelsen's carbol-fuchsin is postulated to be the result of phenol and dye separating out as a liquid phase. Evidence for this point of view is presented. It can be considered that the basis for the acid-fast property lies in the greater solubility of the phenol and dye in the cell constituents than in the decolorizing agent.

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THE INFLUENCE OF "FOLIC ACID" DEFICIENCY IN MACACA MULATTA ON SUSCEPTIBILITY TO EXPERIMENTAL POLIOMYELITIS[1]

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For several years studies have been in progress in this laboratory on the relation of nutrition to resistance to virus infections of the central nervous system. The majority of the reports have involved experiments with mice, but a few have been with monkeys (Clark *et al.* 1945). In the case of the monkey, which requires certain known and unknown components of liver, it has been difficult to produce an uncomplicated deficiency. Thiamine deficiency has been studied by using sulfited liver extract which served as a source of unknown factors. Other work here has shown that a "folic acid" deficiency developed rapidly when our basal synthetic diet was fed along with the synthetic vitamins (Waisman and Elvehjem, 1943). We have therefore studied the possible relation of this deficiency to resistance to experimental poliomyelitis.

EXPERIMENTAL

The methods of handling and feeding the animals have been described previously (Waisman *et al.*, 1943). The basal diet ("folic acid" deficient), consisting of sucrose 73 parts, purified casein 18, mineral salts 4, cod liver oil 3, and corn oil 2, was fed *ad libitum*; and adequate quantities of ascorbic acid, thiamine, riboflavin, nicotinic acid, pyridoxine, calcium pantothenate, choline chloride, *p*-aminobenzoic acid, inositol, and biotin were given daily. The norite eluate concentrate of Hutchings *et al.* (1941) prepared from solubilized liver powder (fraction L)[2] was the source of "folic acid." All these concentrates were assayed by microbiological methods, using *Streptococcus faecalis* R, and were found to contain from 46 to 85 per cent of the original activity of the liver powder. In many instances the animals which were offered this "folic acid" supplement refused to consume it, because of their general weakness and lack of interest in food. In these cases, the vitamin supplement was thickened by adding some basal diet and the whole administered to the monkey by a stomach tube. This process was continued oftentimes for 10 days before the animals were willing to drink the supplement.

Optimum diets contained 3 to 5 per cent of liver extract powder;[2] these were prepared by adding the liver product (3 to 5 per cent) at the expense of the 95 parts of the mixture of dry ingredients.

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[2] Donated by Wilson and Co., Chicago, Illinois.

The procedure developed for the production of "folic acid" deficiency consisted in placing the animals on the basal diet with the known crystalline vitamins until the first constant drop in weight was observed (about 2 months). They were then given the amount of the norite eluate concentrate that would just maintain the weight as determined experimentally. In most cases, however, the animals showed an increased appetite which reflected itself in definite weight gains within a week, and, at this point, the "folic acid" concentrate was discontinued. The animals were then allowed only the basal ration until nutritional failure again resulted. The acute deficiency was considered to be the syndrome following the first or second drop in weight, whereas a state of chronic deficiency was said to exist following 3 or more weight drops necessitating the administration of "folic acid" each time to maintain the animals. Blood studies throughout the periods of depletion have demonstrated the typical progressive leucopenia and the correction of this condition by feeding "folic acid" concentrates.

Since many factors, such as previous nutritional history, genetic variability, age and weight at the start of the experiment, the duration of the experiment, and the amount of "folic acid" fed, influence the response of the individual animal to a "folic acid" concentrate, it was impossible to obtain many animals at a comparable nutritional state at the time of the administration of the virus, even though the animals were placed on experiment at the same time. This was particularly true in the case of the short-term experiments (series 2, 3, 4); for in most cases the first constant drop in weight of the animals occurred after 7 weeks on the deficient ration, and if, as was the case in these series, the virus inoculation was performed during the eighth week, some animals of the group would not yet have shown nutritional failure.

The MV strain of poliomyelitis was used throughout. The supernatant of a 5 per cent infected monkey cord suspension, after overnight sedimentation in an ice chest, was used directly for all intranasal inoculations. These insufflations were made by dropping approximately 1 ml of the virus suspension into each nostril on 3 successive occasions within 24 hours, the first administration being preceded by irrigation of the nasal passages with a M/10 phosphate buffer solution at about pH 5 (Schultz and Gebhardt, 1934). Intracerebral injections were made in the conventional manner under ether anesthesia using as inoculum 1-ml dilutions of this 5 per cent cord suspension, as indicated. The monkeys were observed at least twice daily for signs of illness; the recorded time for the first signs of definite flaccid paralysis was calculated from the third intranasal insufflation. Necropsies were performed on all dead or ether-sacrificed monkeys, and histological studies were made. Bacteriological studies were made whenever indicated, especially in the dysenteries which often complicate "folic acid" deficiency in this animal (Waisman and Elvehjem, 1943). In such cases *Shigella paradysenteriae* was isolated from the stools (table 1). Examination of the intestinal mucosa at autopsy revealed lesions consistent with the diagnosis.

Series 1 (table 1) consisted of 10 monkeys; 4 were maintained on the deficient diet until their condition was of a chronic nature, and 6 were fed the control diets. Following intranasal inoculation 5 of the 6 control animals showed clinical signs of poliomyelitis which progressed to quadriplegia, whereas none

TABLE 1

Influence of "folic acid" on susceptibility of M. mulatta to MV strain poliomyelitis virus

SERIES NO.	MONKEY NO., SEX	WT. (KG)	DIET	VIRUS CONC., ROUTE OF INOCULATION	FIRST SIGNS OF PARALYSIS (DAYS)	PATHOLOGY*	REMARKS
1	16 F	4.1	Optimum	5% I.N.	10	4+	Quadriplegia; approx. 3 mos. pregnant
	144 M	2.7	Optimum	5% I.N.	7	4+	Quadriplegia
	146 F	4.0	Optimum	5% I.N.	9	4+	Quadriplegia
	147	4.1	Optimum	5% I.N.	9	4+	Quadriplegia
	25 F	2.9	Optimum	5% I.N.	7	3+	Quadriplegia
	145	4.6	Optimum	5% I.N.		±	No clinical signs of polio; sacr. 35 days after inoc.
	62 F	3.8	Deficient	5% I.N.		1+	No clinical signs of polio; sacr. 35 days after inoc.
	141 M	2.2	Deficient	5% I.N.		1+	No signs of polio; sacr. 35 days after inoc.
	142 M	3.1	Deficient	5% I.N.		2+	No signs of polio; after 35 days placed on optimum diet; sacr. after 75 days
	143 F	2.5	Deficient	5% I.N.	See series 4		No signs of polio; placed on optimum diet.
2	13 M	5.4	Optimum	5% I.N.	6	3+	Quadriplegia
	105 M	3.0	Optimum	5% I.N.		3+	No frank paralysis; sl. tremors and weakness
	155 M	4.4	Deficient	5% I.N.	7	4+	Quadriplegia
	156 M	3.5	Deficient	5% I.N.	7	4+	Quadriplegia
	154 F	3.2	Deficient	5% I.N.	8	4+	Flaccid par. of both arms
	78 M	2.7	Deficient	5% I.N.		±	Died 4 days after inoc.; clinical dysentery
	159 M	2.4	Deficient	5% I.N.			Died 4 days after inoc.; extensive TB
3	104 M	4.7	Optimum	5% I.N.	6	4+	Quadriplegia
	89 F	3.8	Optimum	5% I.N.	7	4+	Quadriplegia
	162 M	2.7	Deficient	5% I.N.	9	4+	Quadriplegia
	163 F	2.6	Deficient	5% I.N.	7	4+	Quadriplegia
	171 M	2.7	Deficient	5% I.N.	7	4+	Quadriplegia
	172 M	3.0	Deficient	5% I.N.	6	4+	Quadriplegia
	175 F	2.7	Deficient	5% I.N.	8	4+	Quadriplegia
	176 F	4.9	Deficient	5% I.N.	8	4+	Quadriplegia
	177 F	2.2	Deficient	5% I.N.	9	3+	Quadriplegia
	178 M	2.8	Deficient	5% I.N.	7	4+	Quadriplegia
	179 F	4.4	Deficient	5% I.N.	7	3+	Quadriplegia
4	201 F	2.6	Optimum	1:20,000 I.Cer.	12	4+	Quadriplegia
	202	2.0	Optimum	1:20,000 I.Cer.	6	4+	Quadriplegia
	143 F	2.9	Optimum	1:20,000 I.Cer.	7	4+	Quadriplegia

TABLE 1—*Continued*

SERIES NO.	MONKEY NO., SEX	WT. (KG)	DIET	VIRUS CONC., ROUTE OF INOCULATION	FIRST SIGNS OF PARALYSIS (DAYS)	PATHOLOGY*	REMARKS
	185 M	2.7	Deficient	1:20,000 I.Cer.	10	3+	Quadriplegia
	192 M	2.1	Deficient	1:20,000 I.Cer.	9	4+	Quadriplegia
	194	1.9	Deficient	1:20,000 I.Cer.	6	3+	Quadriplegia
	196	2.0	Deficient	1:20,000 I.Cer.	11		Quadriplegia; remained alive 10 days foll. quadriplegia
	198	2.1	Deficient	1:20,000 I.Cer.	9	4+	Quadriplegia
	205 F	1.9	Deficient	1:20,000 I.Cer.		±	No clinical signs of polio; sacr. 35 days after inoc.
7	237 M	2.8	Optimum	5% I.N.	10	4+	Quadriplegia
	238 F	3.0	Optimum	5% I.N.	8	4+	Quadriplegia
	232 M	2.5	Deficient	5% I.N.	13	4+	Flaccid par. of both arms; alive and alert for 17 days foll. paralysis
	233 F	2.5	Deficient	5% I.N.		1+	No clinical signs of polio; sacr. 35 days after inoc.

* Stages of poliomyelitis lesions in *Macaca mulatta* as used in table 1:

± Indeterminate. A few (4 to 6 or 8 per section) focal areas of microglial infiltration; most of the ganglion cells appear normal; some, however, show chromatolysis with margination of Nissl substance, and a few show more marked degeneration with satellitosis of microglial cells and lymphocytes; congestion of vessels with occasional slight hemorrhage. Only an occasional polymorphonuclear leucocyte observed in the sections; no meningitis; no perivascular round cell infiltration.

This picture is considered consistent with early asymptomatic poliomyelitis but is not pathognomonic; in this instance, since poliomyelitis virus was introduced, these lesions are probably due to that virus.

1+ Slight perivascular round cell infiltration especially in medulla; larger amount of microglial infiltration than in the "indeterminate" category; extensive chromatolysis with margination of Nissl substance and eccentric nuclei; necrosis of few ganglion cells with invading microglial phagocytes and lymphocytes but very few polymorphonuclear leucocytes; usually slight hemorrhage.

Animals with this degree of damage have commonly shown tremors, ataxia, and weakness, but no paralysis.

2+ Typical lesions with perivascular cuffing, oedema, easily recognized necrosis of nerve cells with neuronophagia, composed of about equal numbers of microglial cells, lymphocytes, and polymorphonuclear leucocytes. Disappearance of an appreciable number of ganglion cells.

3+ Marked typical lesions with extensive nerve cell necrosis and neurono-phagocytosis; most of the invading cells involved in this process are polymorphonuclear leucocytes. Round cell meningitis.

4+ Overwhelming typical lesions with hardly a normal ganglion cell left at any level of the cord studied. Many polymorphonuclear leucocytes throughout.

of the animals on the deficient ration (nos. 62, 141, 142, and 143) ever showed clinical signs of the infection. After the 35-day observation period following inoculation, 2 of the deficient animals (nos. 62 and 141) were sacrificed, and histological studies of the central nervous system sections from these showed definite, although slight, lesions (1+) of poliomyelitis. Monkeys nos. 142 and 143 were placed on the control ration 35 days following inoculation, and one of them (no. 142) was subsequently sacrificed after 40 days on this diet. Histological studies of cord sections showed more marked lesions (2+) of poliomyelitis than did animals nos. 62 and 141. Monkey 143 was subsequently reinoculated (intracerebral route) as a control in series 4 after 166 days on the control diet; it succumbed to typical clinical poliomyelitis. It is manifestly impossible on the basis of this one animal (no. 143) to determine whether the lack of resistance to the infection following the second inoculation has any immunological basis or whether the change to an optimum diet was the determining factor.

The potency of the virus suspension employed is further demonstrated by the fact that, in addition to the 10 animals in this series, 5 animals in another experiment on a high pyruvate diet (Waisman and McCall, 1944) were inoculated with the same virus at the same time and by the same route; 4 of the 5 animals succumbed to the infection.

Series 2 consisted of 5 deficient animals and 2 controls. The intranasal inoculation was made 8 weeks after the animals were placed on the deficient diet; consequently the deficiency was considered acute, since the first constant drop in weight of all the deficient animals began at or after the time of inoculation. All of the controls and 3 of the 5 deficient animals showed clinical signs of the infection, whereas the remaining 2 deficient animals died on the fourth day following inoculation. At autopsy it was found that one of these two animals died of dysentery and the other animal had an extensive miliary tuberculosis. The results of this series do not show any increased resistance on the part of the deficient animals.

A third series was conducted by intranasal inoculation of 2 control and 9 acutely deficient animals. All the animals, regardless of diet, succumbed to typical clinical poliomyelitis and were sacrificed after quadriplegia.

In the fourth series 6 acutely deficient and 2 control animals were employed. The virus was administered by the intracerebral route, using the dilution indicated. All the control monkeys and all the deficients except no. 205 showed clinical signs of the infection. Monkey no. 205 showed no clinical signs of infection and was sacrificed 35 days following inoculation. Histological studies on central nervous system sections of this animal showed "indeterminate" (±) lesions, suggesting the possibility of abortive poliomyelitis. It is interesting to note that one of the deficient animals (no. 196) was able to remain alert and survive without any special care for 10 days after quadriplegia was first noted.

Series 5 and 6 (15 monkeys) are not included in the table because the dilution of virus employed in series 5 (1:200,000) was too great, and very few animals succumbed to the disease. Series 6 was composed of the surviving animals of series 5, thus making the results difficult to interpret.

Series 7 was set up in an attempt to reproduce the conditions of series 1. Two animals were fed the control diet, and 4 were placed on the basal diet. The difficulty of maintaining monkeys in a chronic state of "folic acid" deficiency over long periods of time was again well brought out by this experiment. A critical weight loss, if not immediately recognized as such, resulted in complicating dysentery and death of the animal in 48 hours even though "folic acid" therapy was immediately instituted. At the time of the intranasal inoculation, therefore, only 2 of the deficient animals were still alive (nos. 232 and 233).

Both controls became paralyzed to the extent of quadriplegia (in 8 to 10 days), whereas only one (no. 232) of the deficient monkeys showed any clinical signs of the infection. This one showed paralysis of the right arm on the thirteenth day and paralysis of the left arm on the following day, and was alive and alert for 17 days after onset of the paralysis; however, microscopic examination of CNS sections showed overwhelming typical lesions (4+). The other deficient monkey (no. 233) showed no clinical signs following inoculation and was sacrificed on the thirty-fifth day; micropathology was 1+. The results of this series are in accord with those of series 1 in that the animals in a chronic state of "folic acid" deficiency demonstrate increased resistance to experimental poliomyelitis.

CONCLUSIONS

The results of these experiments indicate that the rhesus monkey, when deficient in "folic acid," exhibits an increased resistance to poliomyelitis virus when the deficiency is of a chronic nature, but not when the deficiency is of the acute type.

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CETYL PYRIDINIUM CHLORIDE[1]

I. GERMICIDAL PROPERTIES

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Cetyl pyridinium chloride is a cationic detergent which is soluble in water, chloroform, alcohol, and acetone. Aqueous solutions are clear, colorless, and odorless. The agent has a depressant action upon surface tension. This property enhances the value of the germicide because irregular surfaces are uniformly wetted.

The germicidal properties of cetyl pyridinium chloride were first studied in this laboratory. Shelton *et al.* (1939, 1940) described the properties of a series of a quaternary ammonium salts and also the effect of the length of the alkyl chain upon germicidal power. Blubaugh *et al.* (1939, 1940, 1941) briefly described the germicidal activity of cetyl pyridinium chloride. Green and Birkeland (1941) reported studies on the sporicidal activity of this compound. They concluded that the spores of *Clostridium perfringens*, *Clostridium sporogenes*, *Clostridium tetani*, *Bacillus anthracis*, and *Bacillus subtilis* were killed under the conditions of the test. These workers reported a curative effect of the compound on artificially infected chick embryos (Green and Birkeland, 1942; Green, 1944).

Huyck (1945) demonstrated that a 1:4,000 dilution of cetyl pyridinium chloride was "bacteriostatic or bactericidal to bacteria found in the oral cavity." Huyck (1944) also described the physical and chemical properties of cetyl pyridinium chloride and reviewed the literature on the quaternary ammonium salts. Warren *et al.* (1942) found that dilutions of 1:100 or 1:250 produced some reddening of scarified rabbit skin, but that greater dilutions were without harmful action. Subcutaneous injections of a 1:100 dilution in phosphate buffer produced slight edema and reddening at the site of injection, but higher dilutions had no untoward effect. These results indicate that for topical application or for wound cleansing cetyl pyridinium chloride is essentially nontoxic in the concentrations generally used.

It is the purpose of this paper to report the germicidal activity of cetyl pyridinium chloride against a variety of organisms, and the effect of temperature, pH, and presence of protein upon the germicide.

MATERIALS AND METHODS

For the following studies the procedure outlined in Circular no. 198 of the U. S. Department of Agriculture was followed whenever possible. For certain

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[1] Cetyl pyridinium chloride is manufactured and sold under the registered trade name "ceepryn" by the Wm. S. Merrell Company.

[2] The authors wish to acknowledge the technical assistance of Mr. C. W. Botts and Dr. I. W. Gibby.

fastidious organisms and for yeasts, molds, and trichomonads, variations in media were necessary. Tests were conducted at 37 C as well as at 20 C and also in the presence and in the absence of serum. These and other variations are listed in appropriate sections. For many of the common organisms the results listed were obtained from three tests in duplicate. All other organisms were tested until comparable readings were obtained.

TABLE 1

Germicidal activity of cetyl pyridinium chloride aqueous solution

ORGANISM	NO. STRAINS TESTED	AVERAGE CRITICAL KILLING DILUTION IN TERMS OF ACTIVE INGREDIENTS AT 37 C	
		No serum	10% bovine serum
Staphylococcus aureus	5	1:83,000	1:12,500
Staphylococcus albus	1	1:73,000	1:12,000
Streptococcus viridans	1	1:42,500	1:12,000
Streptococcus hemolyticus	2	1:127,500	1:17,000
Neisseria catarrhalis	2	1:84,000	1:13,000
Diplococcus pneumoniae I	1	1:95,000	1:14,000
Diplococcus pneumoniae III	1		1:20,000
Pseudomonas aeruginosa	2	1:5,800	<1:1,000
Klebsiella pneumoniae	2	1:49,000	1:5,500
Corynebacterium diphtheria	1	1:64,000	1:14,000
Mycobacterium phlei	1	1:1,500	1:1,000
Eberthella typhosa	5	1:48,000	1:3,000
Escherichia coli	2	1:66,000	<1:1,000
Proteus vulgaris	2	1:34,000	1:2,000
Shigella dysenteriae	1	1:60,000	1:5,000
Shigella paradysenteriae (Flexner)	2	1:52,000	1:3,500
Shigella paradysenteriae (Hiss)	1	1:49,000	1:2,000
Shigella sonne	2	1:68,000	1:6,500
Lactobacillus acidophilus	1		1:16,500
Brucella abortus	1		1:19,500
Trichomonas vaginalis†	1		1:3,000*
Candida albicans	1	1:37,000	1:3,500
Cryptococcus neoformans	1	1:61,000	1:6,000
Trichophyton mentagrophytes	1	1:36,000	1:3,000
Microsporum canis	1	1:34,000	1:5,000

* Twenty-five per cent human serum.

† We are indebted to Dr. Garth Johnson of the State University of Iowa for a pure culture of this organism.

EXPERIMENTAL

The critical killing dilution (C.K.D.) for any given organism is defined as the highest dilution of germicide that will kill in ten minutes but not in five. Critical killing dilutions of cetyl pyridinium chloride were determined for a wide variety of organisms. For some common organisms C.K.D.'s were determined for two or more strains. The tests were run at 37 C in the presence and in the absence of 10 per cent bovine serum (table 1).

The results demonstrate that cetyl pyridinium chloride is germicidal against a wide variety of organisms. In the absence of serum, critical killing dilutions were high. Only against *Mycobacterium phlei* and *Pseudomonas aeruginosa* were the critical killing dilutions less than 1:30,000. Against several common gram-negative organisms germicidal dilutions ranged from 1:34,000 to 1:68,000, and against several species of cocci the range was from 1:42,000 to 1:127,000. Against some common species of fungi and yeastlike fungi germicidal dilutions ranged from 1:34,000 to 1:61,000.

Although the germicidal activity of cetyl pyridinium chloride was somewhat lower in the presence of serum than in its absence, high dilutions were still germicidal against most organisms. Only against *Escherichia coli* and *Pseudomonas aeruginosa* did the critical killing dilutions drop below 1:1,000. For other gram-negative rods the range of germicidal dilutions was from 1:2,000 to 1:19,000. For several species of cocci the range of germicidal dilutions was from 1:12,000 to 1:20,000, and for the fungi and yeastlike fungi the range was from 1:3,000 to 1:6,000. In the presence of 25 per cent human serum a 1:3,000 dilution of cetyl pyridinium chloride destroyed the flagellate *Trichomonas vaginalis*.

TABLE 2

Effect of temperature upon germicidal activity of cetyl pyridinium chloride against organisms of common major groups

ORGANISMS	CRITICAL KILLING DILUTIONS EXPRESSED IN TERMS OF ACTIVE INGREDIENTS			
	37 C		20 C	
	No serum	10% bovine serum	No serum	10% bovine serum
Staphylococcus aureus	1:110,000	1:11,500	1:67,500	1:6,750
Eberthella typhosa	1:83,500	1:4,000	1:62,500	1:1,300
Shigella sonne		1:5,000		1:2,700
Shigella paradysenteriae (Flexner)		1:2,000		<1:1,000
Escherichia coli		<1:1,000		<1:1,000
Neisseria catarrhalis		1:13,500		1:12,300
Klebsiella pneumoniae		1:3,000		1:1,000
Proteus vulgaris		1:1,000		<1:1,000
Pseudomonas aeruginosa		<1:1,000		<1:1,000

If critical killing dilutions are determined in the presence of serum, the use of pooled beef serum is recommended. The protein content of each new lot should be checked to exclude nonuniform batches. Preliminary experiments demonstrated that the use of human ascitic fluid with a protein content considerably below the level for normal beef serum resulted in much higher critical killing dilutions.

The germicide was tested against several organisms at 37 C and 20 C. *Eberthella typhosa* (Hopkins) and *Staphylococcus aureus* (standard resistance strain) were tested in the presence and in the absence of serum, and other organisms were tested only in the presence of serum (table 2).

At 37 C germicidal dilutions were roughly twice as great as at 20 C. In the presence of serum and at 37 C the results obtained in this test were essentially the same as those reported in table 1. Minor variations occurred, but these are within the experimental error of the method.

The effect of pH upon the germicidal activity of cetyl pyridinium chloride was determined in Prideaux and Ward's universal buffer. The buffer alone had no bactericidal effect at any pH level used in the germicide test. The tests were performed at 37 C in the absence of serum. *Staphylococcus aureus* and *Eberthella typhosa* were used as test organisms.

The results obtained are shown in table 3. The results obtained by Gershenfeld and Milanick (1941) and Gershenfeld and Perlstein (1941) from similar tests on zephiran and triton K-12 are also presented for comparison. It is admitted that comparison of our tests with tests run in another laboratory by other workers may be subject to considerable hazard, but the tests were very similar in character and the results are so striking that the comparison seems worthwhile.

Cetyl pyridinium chloride was germicidal against *Eberthella typhosa* and *Staphylococcus aureus* in high dilutions at all pH levels from 2.0 to 10.0. The germicidal effectiveness of cetyl pyridinium chloride at low pH levels was much greater than the germicidal effectiveness of either zephiran or triton K-12. At pH 5.0 the concentration of zephiran and the concentration of triton K-12 necessary for germicidal action were, respectively, approximately 10 and 50 times as great as the concentration of cetyl pyridinium chloride required for germicidal action. At pH 4.0 no end points were obtained for zephiran and triton K-12. This indicates that the difference between the action of these substances and the action of cetyl pyridinium chloride would be even greater under more strongly acid conditions.

It is evident that a solution which contains 50 ppm (a dilution of 1:20,000) of cetyl pyridinium chloride is a potent germicide at any pH level which is likely to be encountered under conditions of actual usage.

INCONSISTENCIES IN TESTING DETERGENTS

The problem of testing quaternary ammonium salts by the methods outlined for phenol coefficients is not a simple one. As the initiated operator knows, consistent, readable results are not always obtained if tests are conducted without serum.

Fairly uniform results are obtained if the tests are conducted in the presence of animal serum. This procedure decreases the germicidal potency of the quaternary ammonium salts and imposes an arbitrary penalty upon their effectiveness. It is, however, a practical solution to the problem and yields useful information about the effectiveness of the germicide under the conditions of usage. The method does not, however, give a true indication of the bactericidal power of these detergents. The primary difficulties of the test seem to lie in the addition of FDA media to the medication tubes. The broth is precipitated as a colloidal dispersion by the quaternary ammonium salts. The

TABLE 3

Effect of pH on germicidal power of three cationic detergents at 37 C in absence of serum

Compounds	pH	2	3	4	5	6	7	8	9	10
Staphylococcus aureus										
Cetyl pyridinium chloride	Dilution	1:66,000	1:66,000	1:58,000	1:54,000	1:60,000	1:66,000	1:66,000	1:58,000	1:54,000
	ppm	15	15	17	18.5	16.5	15	15	17	18.5
Eberthella typhosa										
Cetyl pyridinium chloride	Dilution	1:40,000	1:36,000	1:24,000	1:24,000	1:36,000	1:38,000	1:40,000	1:36,000	1:32,000
	ppm	25	28	42	42	28	26	25	28	31
Staphylococcus aureus										
Zephiran*	Dilution			<1:5,000	<1:5,000	1:20,000	1:70,000			
	ppm			>200	>200	50	14			
Staphylococcus aureus										
Triton K-12*	Dilution			<1:100	1:100	1:400	1:900†	1:12,000‡	1:20,000	
	ppm			>10,000	10,000	2,500	1,111	83	50	
Eberthella typhosa										
Triton K-12	Dilution			1:100	1:300	1:500	1:900†	1:14,000‡	1:20,000	
	ppm			10,000	3,333	2,000	1,111	71	50	

* Data from Gershenfeld and Milanick, and Gershenfeld and Perlstein.

† pH 7.2.

‡ pH 8.2.

effect of this precipitate on the uniformity and homogeneity of the bacterial suspension has not been determined. Effective and complete contact between all bacteria and the germicide may be prevented by this dispersion of the ingredients of the broth.

Variations in the media from lot to lot, and day to day variations in the resistance of the test organisms, have been cited as causes of nonuniform results. These factors are doubtless important, but they do not account for "skips" nor lack of uniformity between duplicate or triplicate tests performed the same day. It would seem that some more random factor must yet be defined.

Klarmann and Wright (1945) recently offered a semisynthetic medium for use in tests of germicides. It is to be hoped that such media will be studied further and that formulae will be devised which will permit the testing of many common organisms.

In the material which has been presented, tests were repeated until readable results were obtained. Also, serum was added to the medication tubes, both to make the tests more readable and to simulate the conditions encountered in medical usage of the germicide. A comparison with phenol is not justifiable, and the results have been reported as critical killing dilutions rather than as phenol coefficients.

DISCUSSION

The quaternary ammonium salt, cetyl pyridinium chloride, is an excellent nonmercurial germicide. It shows high bactericidal potency against a wide variety of organisms: the gram-positive and gram-negative bacteria, some common pathogenic fungi, and one flagellate were all killed in concentrations far below the toxic level of the compound.

The absence of heavy metals and its potency in the presence of proteins make cetyl pyridinium chloride especially valuable for skin disinfection, wound cleansing, vaginal inserts, etc. Because of its detergency and germicidal potency at room temperature, and at extremes of acidity and alkalinity, the compound has wide application in cleansing and disinfecting utensils.

The reason for the high potency of cetyl pyridinium chloride under acid as well as alkaline conditions is not yet known. Literature has been cited which demonstrates that the quaternary ammonium salts in general are actively germicidal in high dilutions only under alkaline conditions (Gershenfeld and Milanick, 1941; Gershenfeld and Perlstein, 1941). Apparently the germicidal action of cetyl pyridinium chloride does not depend upon the degree of dissociation of the compound.

SUMMARY

Cetyl pyridinium chloride is germicidal in high dilutions against a variety of gram-positive and gram-negative bacteria, certain common pathogenic fungi, and the flagellate, *Trichomonas vaginalis*.

Cetyl pyridinium chloride is germicidal in high dilutions under acid as well as alkaline conditions.

The compound is actively germicidal at room temperature as well as at 37 C.

Cetyl pyridinium chloride is germicidal in moderate to high dilutions in the presence of animal serum.

The nontoxic character of the compound and its bactericidal properties under diverse conditions make it an exceptionally versatile and valuable disinfectant.

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THE EFFECT OF ENVIRONMENTAL CONDITIONS ON PENICILLIN FERMENTATIONS WITH PENICILLIUM CHRYSOGENUM X-1612[1,2]

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When penicillin is produced by submerged fermentation in tanks, many factors influence the course of the fermentation and the penicillin yield. Some of these factors are discussed in other reports from this institution. Tank design and the effect of variations in aeration are described by Stefaniak *et al.* (1946). Gailey *et al.* (1946) report results obtained with various cultures. Koffler *et al.* (1945) give data on the metabolic changes occurring in the medium during fermentation. Other studies include the effect of bacteriostatic agents (Knight and Frazier, 1945) and the role of corn steep liquor in penicillin fermentations (Bowden and Peterson, 1946). In the present paper the effects of variation in temperature, pressure, and carbon dioxide tension are reported. Data on the toxicity of various antifoam agents are also given. A comparison of glucose and lactose as carbohydrate sources in tank fermentations is made.

The experimental work was performed during a period when numerous variations in penicillin yields were obtained because of the instability of the culture (Gailey *et al.*, 1946) and deterioration of the steep liquor. Penicillin yields and chemical changes in the medium varied considerably during the course of the experiments.

EXPERIMENTAL METHODS

The culture used was *Penicillium chrysogenum* X-1612 (Carnegie Institution)· The experimental conditions described by Stefaniak and co-workers (1946) were followed throughout these experiments. The methods for the determination of sugar, ammonia nitrogen, organic nitrogen (soluble nonammonia nitrogen), mycelial nitrogen, and pH are reported by Gailey *et al.* (1946).

The carbon dioxide content of the exhaust air in penicillin fermentations was determined in the following manner: Three to five cu ft of exhaust air, depending on the carbon dioxide concentration, were bubbled through 20 ml of 1 N sulfuric acid in a 35-by-200-mm pyrex test tube (to remove ammonia), then through 20 ml of 1 N sodium hydroxide solution in a 22-by-760-mm absorption column, and finally through a wet test meter. After the gas sample had been passed through the apparatus the column was washed free of sodium hydroxide solution with

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[2] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

carbon-dioxide-free water. The resulting solution, after the addition of 10 ml saturated $BaCl_2$ solution, was titrated to the phenolphthalein end point with standard hydrochloric acid. Blanks were determined in the manner described above. In this case a quantity of air from the service line equal to that of the gas sample was used instead of the exhaust air. The difference between the blank titration and the sample titration represented carbon dioxide produced by the culture.

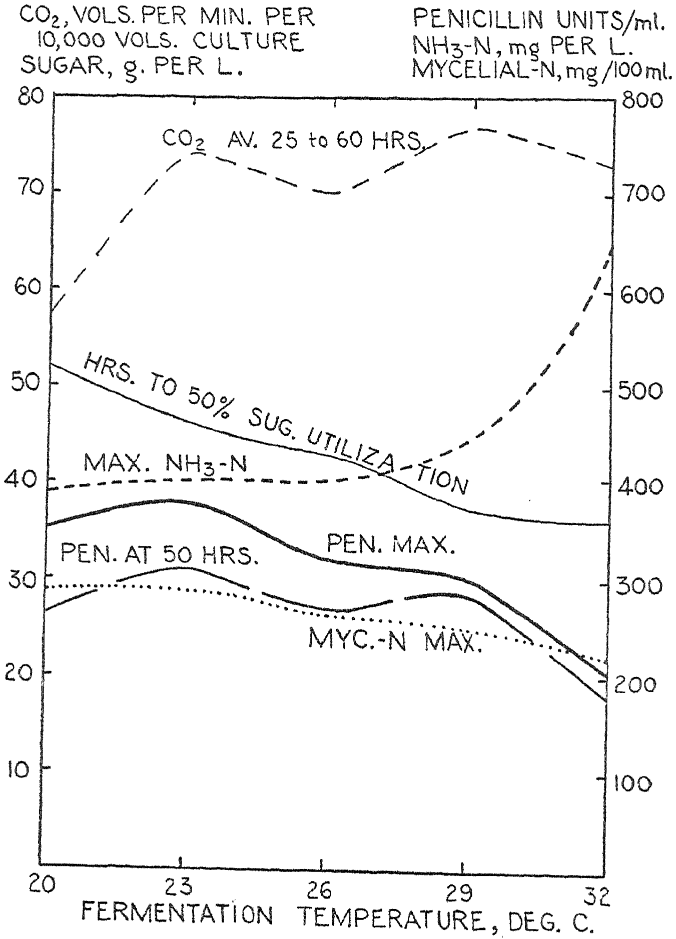

FIG. 1. COMPARISON OF FERMENTATIONS AT VARIOUS TEMPERATURES

EXPERIMENTAL RESULTS

Influence of temperature on penicillin fermentations. In order to determine the effect of temperature on chemical changes within the medium and on penicillin production, tank fermentations were run at 20 C, 23 C, 26 C, 29 C, and 32 C.

These experiments were performed during a period when considerable difficulty was encountered with culture variation and when penicillin yields of only 250 to 350 units per ml were being obtained. The composition of the medium used was as follows: lactose 3 per cent, steep liquor solids 4 per cent, and calcium carbonate 1 per cent. Figure 1 summarizes the data for these experiments. The values given represent 2 fermentations at 20 C, 3 fermentations at 26 C, and 4 fermentations at each of the other temperatures. When the temperatures of penicillin fermentations were raised, the rate of sugar utilization and the rate of carbon dioxide production increased. It is apparent that the culture utilized the available nutrients more rapidly at the higher temperatures. The ammonia nitrogen maximum, which usually occurs at 18 to 24 hours, was not increased appreciably until the incubation temperature was raised to 32 C. The high ammonia maximum at this temperature was correlated with the fact (not shown in figure 1) that, although rapid oxidation of steep liquor nitrogen compounds occurred, growth during the initial period was slow; therefore less ammonia was assimilated. When the fermentations were conducted at temperatures above 26 C, autolysis occurred before the fermentations were terminated because of the exhaustion of the available nutrients. The pH and the ammonia content of the

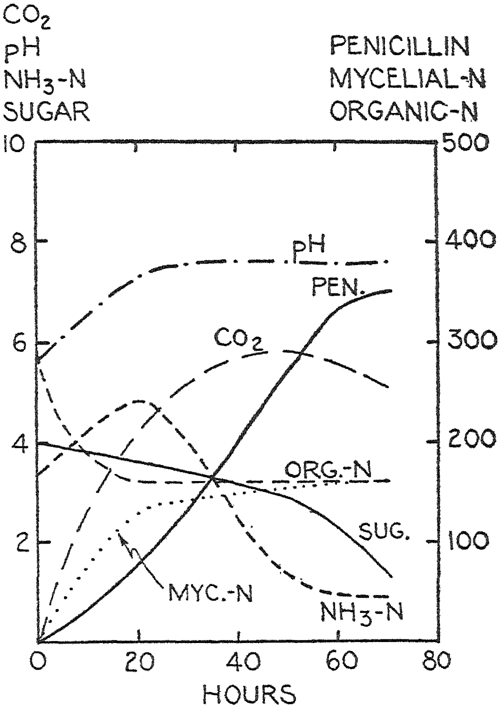

FIG. 2. PENICILLIN FERMENTATIONS AT 2-LB PRESSURE PER SQ INCH
(Values are averages from 4 fermentations.)
CO_2, vols. per minute per 1,000 vols. culture; NH_3-N, mg per 10 ml; sugar, grams per 100 ml; penicillin, units per ml; mycelial-N, mg per 100 ml; organic-N, mg per 100 ml.

medium rose very rapidly when autolysis occurred. Penicillin yields were not appreciably affected at temperatures below 32 C. The maximum yield at this temperature was considerably below the maximum at any other temperature. With the exception of the 32 C fermentations, the penicillin yields at 50 hours were similar. The amount of penicillin produced after 50 hours in fermentations conducted at 29 C and 32 C was very small.

Effect of pressure on the fermentation. A series of experiments in which the internal pressure of the tanks was maintained at 2, 20, and 40 lb per sq inch gage pressure were performed. The data are summarized in figures 2, 3, and 4. The medium used in these experiments contained 4 per cent lactose, 4 per cent steep liquor solids, and 1 per cent calcium carbonate, except in one 20-lb run in which 3 per cent lactose was used. The rate of metabolism (rate of carbon dioxide production) was higher at the 20- and 40-lb pressures; nevertheless the sugar utilization was about the same in all cases. The pH plateau was lowered at the higher pressures. The observed pH change with pressure approximates that calculated from the increase in partial pressure of carbon dioxide at the higher tank pressures. The ammonia maximum was depressed with increasing pressure. At 40-lb pressure the amount of ammonia nitrogen present after

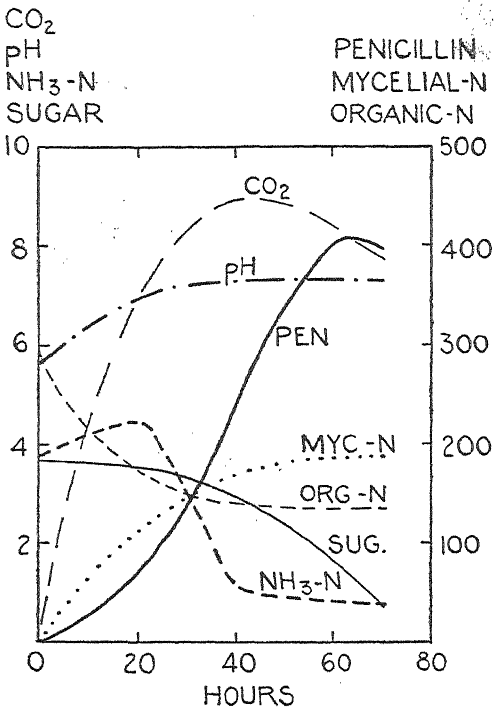

FIG. 3. PENICILLIN FERMENTATIONS AT 20-LB PRESSURE PER SQ INCH
(Values are averages from 3 fermentations.)
CO_2, vols. per minute per 1,000 vols. culture; NH_3-N, mg per 10 ml; sugar, grams per 100 ml; penicillin, units per ml; mycelial-N, mg per 100 ml; organic-N, mg per 100 ml.

50 hours was very low and may have limited penicillin formation. Somewhat heavier growth occurred at the higher pressures. The highest penicillin yields were obtained at 20-lb pressure, although a yield in excess of 400 units per ml has been obtained at 2-lb pressure. The penicillin yields at 40-lb pressure were consistently low.

In summary, it may be concluded that pressure has but little effect on penicillin production, except that at high pressures the slow pH rise decreases the rate of penicillin formation during the early stages of the fermentation, and the low ammonia levels present at the later stages may limit penicillin formation.

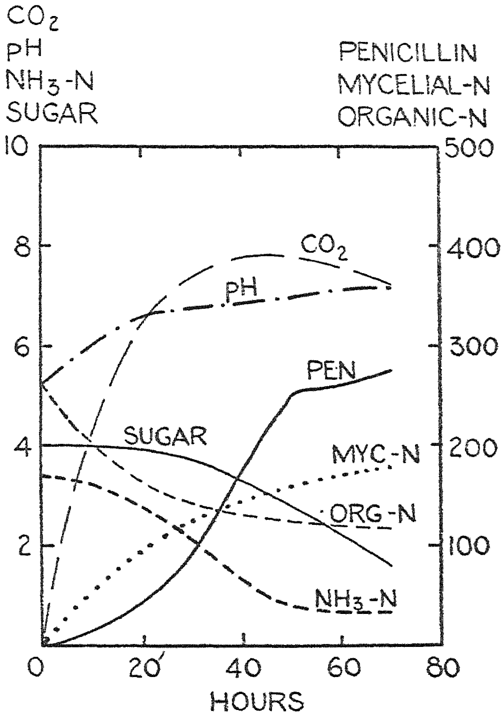

FIG. 4. PENICILLIN FERMENTATIONS AT 40-LB PRESSURE PER SQ INCH
(Values are averages from 4 fermentations.)
CO_2, vols. per minute per 1,000 vols. culture; NH_3-N, mg per 10 ml; sugar, grams per 100 ml; penicillin, units per ml; mycelial-N, mg per 100 ml; organic-N, mg per 100 ml.

Effect of carbon dioxide tension on the fermentation. In industrial penicillin fermentations in large tanks the carbon dioxide content of the medium and the exhaust air are usually higher than in our tanks. This occurs because at low aeration rates the efficiency of air utilization is higher (Stefaniak *et al.*, 1946, figure 10), and because the greater depth of medium results in better air utilization. High carbon dioxide tensions also occurred in the medium in our high-pressure fermentations. In order to determine whether high carbon dioxide levels had any effect on the fermentations other than a lowering of pH, fermentations were set up in which the air used for aeration contained 2 per cent carbon dioxide. This resulted in a carbon dioxide content of from 2.5 per cent to 3.0 per cent in the exhaust air. The penicillin yield, the rate of carbon dioxide production, and the chemical changes in these fermentations (except for a somewhat lower pH level) were similar to those of a typical fermentation (figure 3). It is apparent that increased carbon dioxide tension in the medium does not affect penicillin fermentations.

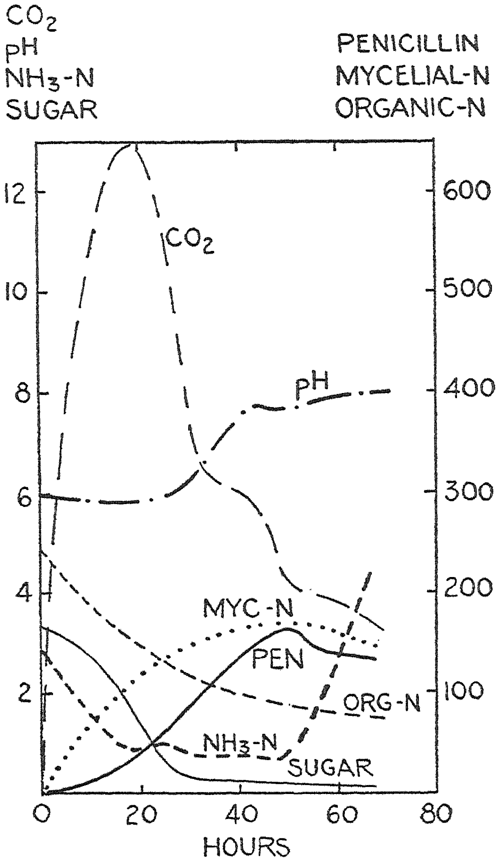

FIG. 5. A PENICILLIN FERMENTATION WITH GLUCOSE AS THE CARBOHYDRATE SOURCE IN THE MEDIUM*

CO_2, vols. per minute per 1,000 vols. culture; NH_3-N, mg per 10 ml; sugar, grams per 100 ml; penicillin, units per ml; mycelial-N, mg per 100 ml; organic-N, mg per 100 ml.

Glucose as a carbohydrate source. Several fermentations were performed to determine whether good penicillin yields could be obtained when glucose is used in the fermentation medium as a carbohydrate source. Figure 5 summarizes the data for one of these experiments. The rate of carbon dioxide production was high during the initial 26 hours of the fermentation and then decreased very rapidly. The glucose and the available ammonia nitrogen were completely utilized in 30 hours. After the disappearance of available carbohydrate and ammonia nitrogen, the nutrients furnished by the steep liquor solids permitted continued growth. When this source of nutrient material could not meet the demands of the culture, autolysis occurred. During autolysis the ammonia nitrogen content and the pH of the medium rose and the mycelium nitrogen decreased. Penicillin formation stopped when autolysis began. Ammonia assimilation during glucose utilization took place at a more rapid rate than ammonia formation from steep liquor nitrogen compounds. Since the ammonia concentration decreased rather than increased, the pH rise was delayed. The increase in pH after glucose exhaustion may be attributed to lactate oxidation (Koffler *et al.*, 1945).

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* The constituents of the medium were as follows: glucose 40 grams, steep liquor solids 40 grams, calcium carbonate 10 grams, and water to 1 liter.

It may be concluded that the superiority of lactose over glucose as a carbohydrate source lies in its slow availability to the culture. In lactose fermentations, ammonia formation from steep liquor nitrogen compounds and lactate utilization bring about an early pH rise. Autolysis is delayed because of the long period required for complete lactose oxidation. On the other hand, in glucose fermentations the pH value during glucose oxidation is too low for rapid penicillin formation, and the early occurrence of autolysis limits penicillin production after exhaustion of the glucose.

Toxicity of antifoam agents. A number of shake-flask experiments were conducted to test various antifoam agents for toxicity. Table 1 summarizes the data for two toxicity experiments conducted in shake flasks. As may be seen, small amounts of any antifoam agent increased the yield over the controls, except in the case of vegifat Y. This has been found generally true of antifoam agents in shake-flask experiments and is apparently the result of the increased oxygen supply when no foam is present in the flask. When penicillin yields are considered, lard, lard oil, and 3 per cent octadecanol in lard oil were not toxic at any of the concentrations used. However, the pH levels were progressively lowered with increasing concentrations of these antifoam agents. It will be noticed that the decrease in pH level follows the degree of toxicity very closely. The foam-breaking capacity of the antifoam agents tested varied considerably. Nopco defoamer and vegifat Y were the most effective foam-breaking agents and were also the most toxic. Three per cent octadecanol in soybean oil was toxic in shake-flask fermentations, nevertheless this same material was not toxic when used as an antifoam agent for tank fermentations.

The antifoam agent regularily used to control foam in our tank fermentations was 3 per cent octadecanol in lard oil. Before the installation of a good method for antifoam agent addition, several fermentations were completed in which 4 liters of this material were added during the first 5 to 10 hours of the fermentation. The pH rise and the carbon dioxide maximum were delayed. In these fermentations, pH 7.0 was generally reached at 35 to 40 hours.

Of all the antifoam agents tested 3 per cent octadecanol in lard oil appeared to be the most useful. However, no systematic study of antifoam agents has been attempted with pilot plant equipment.

TABLE 1

Toxicity of antifoam agents in shake-flask fermentations

ANTIFOAM USED	CONC. ANTI-FOAM PER CENT	4 DAYS		5 DAYS		6 DAYS		7 DAYS		MAX. PEN. U/ML	DAY
		pH	Pen. u/ml	pH	Pen. u/ml	pH	Pen. u/ml	pH	Pen. u/ml		
None		7.58	88	8.35	90			8.50	76	90	5
Lard oil	0.1	7.59	94	8.14	112			8.48	99	112	5
	0.3	7.66	108	8.02	110			8.56	108	110	5
	1.0	7.35	87	7.68	110			7.86	124	124	7
Soybean oil	0.1	7.64	89	8.37	98			8.49	80	98	5
	0.3	7.40	86	7.79	80			8.40	106	106	7
	1.0	6.94	61	7.07	52			7.15	68	68	7
3% Octadecanol in soybean oil	0.1	7.81	94	8.41	95			8.42	76	95	5
	0.3	7.19	83	7.96	96			8.43	92	96	5
	1.0	7.13	64	7.41	46			7.48	67	67	7
None		7.84	71	7.48	75	7.68	65	7.91	97	97	7
3% Octadecanol in lard oil	0.1	7.48	86	7.62	94	7.27	79	7.89	101	101	7
	0.3	7.47	81	7.83	91	7.19	102	7.70	127	127	7
	1.0	7.20	73	7.37	86	7.47	80	7.40	133	133	7
Vegifat Y	0.1	7.39	39	7.54	59	7.64	62	8.26	89	89	7
	0.3	6.10		6.64	33	7.12	51	7.29	76	76	7
	1.0					4.81	0	5.08	7	7	7
Lard	0.1	7.46	60	7.24	90	7.66	88	8.03	118	118	7
	0.3	7.41	91	7.30	92	7.57	98	7.74	120	120	7
	1.0	7.30	81	6.92	108	7.32	126	7.38	146	146	7
Nopco defoamer	0.1	7.63	78	7.64	94	7.93	98	7.70	121	121	7
	0.3	7.43	72	7.48	81	7.60	86	7.83	116	116	7
	1.0	5.60		5.86	16	6.91	36	6.84	86	86	7
Corn oil	0.1	7.47	84	7.65	89	8.15	103	8.11	142	142	7
	0.3	7.40	80	7.44	88	7.98	108	8.01	123	123	7
	1.0	7.07	89	7.08	101	7.21	94	7.20	107	107	7

Five-hundred-ml Erlenmeyer flasks containing 100 ml of medium were incubated in a reciprocating shaker (4-inch stroke, 90 cycles per minute at 23 C). Each flask was inoculated with 5 ml of vegetative inoculum grown on 6 per cent dextrin +2 per cent steep liquor solids medium. The medium used for the toxicity tests was as follows:

Lactose	20.0 grams
Steep liquor solids	20.0 grams
$CaCO_3$	2.0 grams
$NaNO_3$	1.5 grams
KH_2PO	0.25 grams
$MgSO_4 \cdot 7H_2O$	0.12 grams

Distilled water to 1 liter

Each number represents an average value for 3 flasks. The double lines separate experiments performed 1 week apart.

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SUMMARY

Penicillin yields were not appreciably affected by incubation temperatures ranging from 20 C to 29 C, but at 32 C, yields were definitely lower. Metabolic processes were more rapid at higher temperatures. Fermentations were usually conducted at a tank pressure of 20 lb per sq inch. Reducing this pressure to 2 lb per sq inch did not affect the fermentation, but at a tank pressure of 40 lb per sq inch, penicillin yields were reduced. Increased carbon dioxide tension did not affect penicillin fermentations. The metabolic changes in fermentations in which glucose was the carbohydrate source were compared with those on standard lactose medium. Toxicity tests of antifoam agents in shake-flask experiments have been performed. Of the agents found to be nontoxic, 3 per cent octadecanol dissolved in lard oil has been found to be the best agent for controlling foam in tanks.

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A COMPARISON OF PENICILLIN-PRODUCING STRAINS OF PENICILLIUM NOTATUM-CHRYSOGENUM[1]

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Most of the work reported in this paper was done under a government-sponsored co-operative research program. One of the problems at this laboratory involved a comparison of the various strains of the *Penicillium notatum-chrysogenum* group which have been isolated or developed in co-operating laboratories.

EXPERIMENTAL METHODS

Sampling and storage. The fermentations were conducted in the 100-gallon tanks described by Stefaniak *et al.* (1946). Samples were removed periodically into 500-ml flasks and filtered. A 0.5-ml filtered sample was immediately diluted for penicillin assay by addition to previously chilled phosphate buffer in volumetric flasks (4.0 g KH_2PO_4 and 1.0 g K_2HPO_4 per 500 ml solution in distilled water; pH 5.9 to 6.0). These flasks were stored at 10 C until they were assayed for penicillin (usually no longer than 16 hours). Samples kept 2 days showed no change in potency greater than the variations to be expected in the assay, ca. 5 to 10 per cent. A 1.0-ml filtered sample was diluted to 10.0 ml with 0.1 N H_2SO_4 and frozen until sugar determinations and Kjeldahl nitrogen determinations could be made. The remainder of each filtered sample was also frozen and saved for the ammonia determination. The pH values were determined on the original unfiltered samples by means of a glass electrode.

Penicillin assay. The cylinder plate assay for penicillin (Abraham *et al.*, 1941; Schmidt and Moyer, 1944) was used with modifications. *Staphylococcus aureus* 209-P was used as the test organism. Eight plates were set up for each two samples and the results averaged. The standard penicillin was checked against a Food and Drug Administration standard (penicillin G).

Sugar determination. The lactose in diluted samples was hydrolyzed by heating to 120 C for 30 minutes in 0.75 N HCl. Sugar analysis was conducted by the method of Shaffer and Somogyi (1933). Reagent 50 with 5 grams of KI was used, and the samples were heated for 30 minutes in the boiling water bath. Glucose and lactose values were determined from appropriate calibration curves.

Total nitrogen. The total nitrogen of the filtrates was also determined on the diluted samples by the micro-Kjeldahl method described by Johnson (1941).

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Duplicate tubes containing 0.01 ml of filtrate were analyzed. From the values thus obtained were calculated the mycelial nitrogen (by difference from the zero time sample) and the organic nitrogen of the medium (by subtracting the ammonia nitrogen values).

Ammonia nitrogen. Samples of undiluted filtrate were analyzed for ammonia nitrogen by a modification of the aeration procedure described by Umbreit and Bond (1936). A bank of 18-by-150-mm test tubes was constructed in sets of two tubes, one for the sample and one for the acid. The addition of a few drops of water-white caprylic alcohol (practical *n*-octyl alcohol, Eastman Kodak Company) was necessary to prevent frothing of the samples. Since the aeration is considerably slower in this size tube than in those used by Umbreit and Bond, a higher pH was used to hasten the liberation of ammonia. An excess of alkali (1 ml of 10 N NaOH) was used, and aeration at a rate of 40 to 50 ml per minute continued at least 15 hours at room temperature. It is known that this alkalinity liberates easily hydrolyzable ammonia (as in certain amides) as well as ammonia from salts. The results, therefore, represent free and loosely combined ammonia. In the case of the unfermented steep liquor this combined ammonia is roughly 40 per cent of the total ammonia, 60 per cent occurring as free ammonium salts.[2] Two 5.0-ml aliquots of each sample were analyzed. The receiving acid was approximately 0.09 N H_2SO_4 containing methyl red. After aeration the tubes and several blanks were titrated with 0.0357 NaOH (hence, when using a 5.0-ml sample, the titration difference multiplied by 10 represents mg ammonia nitrogen per 100 ml of medium).

Preparation of the inoculum. Soil stocks of the various cultures were kept, and spore plates in 6-oz bottles were made by inoculating small amounts of soil onto the agar surface and moistening slightly with sterile water. The sporulation medium, developed at the Northern Regional Research Laboratory, consisted of the following ingredients (in grams per liter):

Glycerol	7.5
Brer rabbit molasses (orange label)	7.5
Curbay BG (U.S.I.)	2.5
Peptone	5.0
$MgSO_4 \cdot 7H_2O$	0.05
KH_2PO_4	0.06
NaCl	4.0 or 40.0
Agar	25.0

Table 1 shows the effect of temperature and NaCl concentration on the sporulation of several cultures. Peptone is required for mycelium formation, and the NaCl concentration is important for sporulation at 30 C but not at 23 C. The omission of any one constituent, other than peptone, has no effect in retarding sporulation. After incubation at 23 to 25 C for 4 days (or, for certain cultures, 30 C with the high salt medium), the plates were stored at 10 C for as long as 2 weeks before use. The inoculum for tank fermentations was grown in seed tanks as described by Stefaniak *et al.* (1946). Two methods of inoculating this seed tank have been used. The earlier method involved adding an aqueous suspension of spores from two 6-oz bottle plates to the 68 liters of medium and incubating at 23 C for at least 48 hours. The later method made use of 200 ml of vegetative inoculum grown in two 500-ml flasks on a shaker at 23 C. This usually overcame the variable induction period prior to good mycelium formation and, hence, resulted in a more reproducible inoculum.

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[2] It has been found that a more exact liberation of ammonia from the ammonium salts alone can be accomplished by 24 hours' aeration at 40 to 50 ml per minute at pH 9.7 to 10.1 (0.5 ml saturated Na_2CO_3 per 5.0 ml sample).

TABLE 1

Sporulation of cultures

(The constituents of the medium are listed in the text under Preparation of the inoculum)

CULTURE	23 C INCUBATION			30 C INCUBATION		
	Concentration of NaCl			Concentration of NaCl		
	0%	0.4%	4.0%	0%	0.4%	4.0%
832		+	+		+	+
1951-B25		+	+		—	+
1982		—	(+)		(+)	(+)
23248		+	+		—	+
25099		+	+		—	+
35217		+	+		—	+
35347		+	+		—	+
45417		+	+		—	+
R-38		+	+		+	+
J347		+	+		+	+
X-1612	+	+	+	—	—	+

— indicates no formation of green spores.
(+) indicates formation of gray or poor spores.
+ indicates formation of green spores in 3 to 4 days.

Cultures. A list of the cultures mentioned in this report is presented in table 2.[3]

Fermentation equipment. In an attempt to test cultures under conditions similar to those in tanks, 4-liter fermentations were conducted in 9-liter bottles, which were arranged for aeration (17 to 20 liters per minute through 25 to 35 $\frac{1}{32}$-inch holes), for stirring (two propeller blades, 5 inches in diameter, rotating at 250 rpm), for the addition of antifroth (30 to 50 ml of 3 per cent octadecanol in lard oil), and for sampling under aseptic conditions. The spargers, propellers, and sampling tubes were made of aluminum alloy and fitted through an iron cap, which was packed with cotton when placed on the bottles before sterilization.

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[3] Acknowledgment for these cultures is gratefully given to Dr. K. B. Raper of the Northern Regional Research Laboratory, Peoria, Illinois; Dr. G. W. Beadle of Stanford University, California; Dr. C. M. Christensen of the University of Minnesota, St. Paul, Minnesota; Dr. M. Demerec of the Carnegie Institution, New York; and Dr. M. P. Backus of the University of Wisconsin, Madison, Wisconsin.

The medium was inoculated with 8 to 10 per cent of vegetative inoculum, which was grown in shaken flasks in a liquid medium containing 2 per cent steep liquor solids and 6 per cent dextrin (dextrin 151, Clinton Company, Clinton, Iowa). The bottles were incubated in a water bath at 23 C. The 200-liter fermentations were conducted in the 100-gallon tanks described by Stefaniak *et al.* (1946).

TABLE 2

List of Penicillium cultures tested

DESIGNATION	SOURCE	DESCRIPTION
832	N.R.R.L.	*Penicillium notatum*, isolated from natural source
1951-B25	N.R.R.L.	*P. chrysogenum*, single spore isolated from 1951-B, a sector of 1951 which was isolated from a cantaloupe
1982	Stanford	*Penicillium*, ultraviolet mutant from NRRL1950
3271	Standord	*P. notatum*, ultraviolet mutant from NRRL832
4171	Stanford	*P. notatum*, ultraviolet mutant from NRRL832
23248	Stanford	*P. chrysogenum*, ultraviolet mutant from NRRL1951-B25
25099	Stanford	*P. chrysogenum*, ultraviolet mutant from NRRL1951-B25
35217	Stanford	*P. chrysogenum*, ultraviolet mutant from NRRL1951-B25
35347	Stanford	*P. chrysogenum*, ultraviolet mutant from NRRL1951-B25
45417	Stanford	*P. chrysogenum*, ultraviolet mutant from NRRL1951-B25
R-13	Minnesota	*Penicillium*, isolated from dried soil
15-U-1	Minnesota	*Penicillium*, ultraviolet mutant from R-13
R-38	Minnesota	*Penicillium*, isolated from dried soil
R-1138	Minnesota	*Penicillium*, isolated from soil
R-1139	Minnesota	*Penicillium*, isolated from soil
R-1204	Minnesota	*Penicillium*, isolated from soil
R-1205	Minnesota	*Penicillium*, isolated from soil
R-1262	Minnesota	*Penicillium*, isolated from soil
X-1612	Carnegie	*P. chrysogenum*, X-ray mutant from NRRL1951-B25
J347	Wisconsin	*Penicillium*, isolated from a natural source
Q176	Wisconsin	*P. chrysogenum*, ultraviolet mutant from X-1612
Q176-A8	Wisconsin	*P. chrysogenum*, single spore isolated from Q176

CONDITIONS FOR CULTURE COMPARISON

In order to find mold cultures which produce large amounts of penicillin, it became necessary to test a large number of strains of the *Penicillium notatum-chrysogenum* group. Co-operating workers at this and other universities conducted many preliminary small-scale tests using various techniques to separate poor penicillin-producing cultures from possible good producers. In this laboratory experiments were confined to shaken flasks, 9-liter stirred and aerated bottles, and 100-gallon tanks. The cultures which reached the shaken-flask test were tried under a variety of conditions, but none gave better yields than the control organism (*Penicillium notatum* NRRL 832) in the early tests. It was realized that conditions in flasks were not comparable with those in large tanks (Koffler *et al.*, 1945; Bowden and Peterson, 1946). Fortunately, 9-liter stirred and aerated bottles were found to give good yields in 5 to 6 days, and the conditions were more like those in tanks. A number of cultures were compared by this method, and the results, shown in table 3, indicate the superiority of some cultures over others. The best strains appeared to be *Penicillium* strains 45417 and X-1612. It was possible to test most of these superior cultures in the 100-gallon tanks.

Conditions for tank fermentations. Some of the characteristics and conditions of tank fermentations have been discussed by Stefaniak *et al.* (1946). The question of deciding upon standard conditions for comparing cultures raises the problem of the suitability of any one medium for all cultures. It is to be expected that certain characteristics (such as temperature range, pH range, general nutrient requirements) of all strains of the *Penicillium notatum-chrysogenum* group would be similar. It is probable, however, that specific differences between strains would cause the strains to differ widely in their optimum conditions for penicillin production. During the early experiments variations were made in the media between parallel tank fermentations in order to determine the most probable optimum conditions. Chemical changes (particularly lactose

TABLE 3

Penicillin yields of various cultures in stirred bottle fermentations

CULTURE	MEDIUM I		MEDIUM II	
	Penicillin yield	Age at maximum yield	Penicillin yield	Age at maximum yield
	units/ml	*days*	*units/ml*	*days*
832	90	7	145*	6
1951-B25			156	5
1982	166†	8	183	
3271	153	5		
4171	91	6		
23248	134	6		
25099	58	5	194	5
35217	194†	6	148	6
35347	169†	6	207	6
45417	159†	6	267*	6
R-13	140	5		
15-U-1			203	5
R-38	181	5	158	5
R-1138	127	4	160	4
R-1139			217	4
R-1204	168	4	226	5
R-1205			227	6
R-1262	135	5	102	5
J347	96	5	100	5
X-1612			294	6

Medium I contained 2% lactose, 2% steep liquor solids, and salts (1.5 g $NaNO_3$, 1.5 g $CaCO_3$, 0.25 g KH_2PO_4, and 0.125 g $MgSO_4 \cdot 7H_2O$ per liter).

Medium II contained 3% lactose, 4% steep liquor solids, and 1% $CaCO_3$.

* One bottle; all other figures refer to the average yield of 2 or 3 bottles.

† Medium I with no salts except 2.5 g $CaCO_3$ per liter.

and ammonia nitrogen) were determined in order to ascertain roughly the metabolic characteristics of the cultures and the nutrient conditions throughout the fermentations.

The information on carbohydrate metabolism and its relation to the changes in ammonia concentration and pH value, which has been discussed by Koffler *et al.* (1945), was exemplified repeatedly in the tank fermentations. Thus glucose was found to be unsuitable for a comparison of cultures because it was utilized too rapidly; this resulted in a low pH value early in the run, rising too slowly for good penicillin production, a deficiency of available ammonia nitrogen in the penicillin-producing phase, and an early autolysis of mycelium on exhaustion of the carbohydrate. However, the ammonia level in some lactose fermentations tended to rise too high; this condition could be corrected by the addition of a small amount of glucose to the medium in order to stimulate mycelial growth. The addition of glucose was accomplished by inoculating the tanks with 10 per cent inoculum containing 1 to 2.5 per cent of unfermented glucose. At least 3 per cent lactose, and later 4 per cent, was used in the fermentation medium in order to provide sufficient carbohydrate for a 72-hour fermentation.

TABLE 4

Effect of concentration of steep liquor solids on penicillin production by P. chrysogenum X-1612 in tank fermentations

RUN NO.	STEEP LIQUOR SOLIDS	PENICILLIN YIELD	AGE AT MAXIMUM YIELD
	%	*units/ml*	*hours*
138	2*	235	54
139	2*	240	45
118	4†	479	64
119	6†	525	55
116	6†	636	67

* The medium contained 2 per cent steep liquor solids, 2 per cent lactose, and salts (1.5 g $NaNO_3$, 2.0 g $CaCO_3$, 0.25 g KH_2PO_4, 0.125 g $MgSO_4 \cdot 7H_2O$ per liter).

† The medium contained 3 per cent lactose and 1 per cent $CaCO_3$ besides the steep liquor solids.

The concentration of steep liquor in the medium has a marked effect on the fermentation (table 4). It will be seen that there is a progressive increase in yield with an increase in steep liquor concentration (probably due to better growth). The limit may not have been reached but there are disadvantages in using the higher concentrations (e.g., frothing is an important problem in fermentations of the higher concentrations of steep liquor). It was decided that a comparison of cultures on 4 per cent steep liquor solids would be most useful.

Two types of steep liquor have been tested. Table 5 gives a comparison of the regular steep liquor (Staley Manufacturing Company, Decatur, Illinois) and a fermented steep liquor (Corn Products Refining Company, Argo, Illinois). The major differences between the two steep liquors are in their content of reducing material and lactic acid. The unfermented steep liquor is low in lactic acid and high in reducing sugars. The amount of lactic acid in the fermented steep liquor above that in the regular steep liquor is roughly equivalent to the loss in reducing material calculated as glucose. Thus the loss of sugar does not change the amount of available carbon but results, in the fermentation, in the liberation of more ammonia in the fermented liquor runs than in the regular runs. The subsequent drop in ammonia nitrogen is delayed or incomplete in the fermented steep liquor runs. Hence, the pH value is usually higher in these runs. The penicillin yields are usually higher in the regular steep liquor fermentations; hence, regular, unfermented steep liquor was chosen for the standard comparisons.

TABLE 5

Comparison of corn steep liquors

(*P. chrysogenum* X-1612 grown on 3 per cent lactose, 4 per cent steep liquor solids, and 1 per cent $CaCO_3$)

REGULAR STEEP LIQUOR							FERMENTED STEEP LIQUOR						
Run	Penicillin yield	pH at maximum yield	Ammonia maximum	Age at ammonia maximum	Ammonia minimum	Age at ammonia minimum	Run	Penicillin yield	pH at maximum yield	Ammonia maximum	Age at ammonia maximum	Ammonia minimum	Age at ammonia minimum
no.	*units/ml*		*mg/100 ml*	*hours*	*mg/100 ml*	*hours*	*no.*	*units/ml*		*mg/100 ml*	*hours*	*mg/100 ml*	*hours*
126	558	8.1	34	24	26	48	128	415	8.0	43*	23	7	47
							129	330	7.8	43*	23	7	47
130	405	7.5	39	24	15	48	132	370	7.6	60	24	21	46
131	405	7.3	26	24	15	48	133	375	7.8	51	24	11	77
164	440	7.4	37	25	6	48	162	225	7.9	92	31	40	66
165	365	7.5	35	25	7	48	163	410	7.8	71	31	27	66
206	240	7.8	50	26	7	50	212	295	7.9	86	19	35	48
207	250	7.5	50	20	8	50	213	175	8.2	88	19	39	72
142†	312	7.1	40	50	10	68	144†	239	7.6	91	24	14	72
143†	300	7.4	47	35	10	68	145†	260	7.7	90	24	25	72

Reducing material (as glucose) in regular steep liquor solids, 85 to 140 mg per g; in fermented steep liquor solids, 10 to 15 mg per g. Lactic acid in regular steep liquor solids, 130 to 140 mg per g; in fermented steep liquor solids, 230 mg per g.

* In these runs the inoculum contained 4.5 per cent glucose when used. The ammonia concentrations are therefore lower.

† These fermentations were aerated at 30 liters per minute instead of 200 liters per minute and demonstrate the slower growth and utilization of ammonia at this aeration rate.

There are many factors which affect the pH of a fermentation. A comparison of NaOH and $CaCO_3$ as neutralizing agents is given in table 6 for several cultures. It will be seen that the $CaCO_3$ medium usually gave higher yields, earlier penicillin maxima, and lower pH values than the NaOH medium. For these reasons $CaCO_3$ at a level of 1 per cent was used regularly.

In temperature experiments with shaken flasks a temperature optimum around 23 C was observed. A comparison of several temperatures in tank fermentations showed that the optimum range was rather broad. A temperature of 20 C retarded the fermentation but normal yields were eventually obtained. Temperatures from 23 C to 32 C were tested and found to give variable results; sometimes the higher temperatures gave rise to rapid fermentations which resulted in lower yields, but occasionally a good yield was observed even at 32 C. Nevertheless, a temperature of 23 C was used for all standard fermentations.

In brief, the choice of a standard medium for comparison of cultures depended on the ability of the medium to maintain good penicillin-producing characteristics: rapid mycelial growth, presence of a slowly fermentable sugar, presence of ammonia nitrogen during lactose utilization, pH values from 7.0 to 7.8 during the penicillin-producing phase, and late initiation of autolysis. The medium best suited to meet these requirements seems to be 3 to 4 per cent lactose, 4 per cent steep liquor solids, and 1 per cent $CaCO_3$. Ten per cent inoculum grown on 4 per cent glucose, 2 per cent steep liquor solids, 0.5 per cent $CaCO_3$, and salts (Stefaniak *et al.*, 1946) was used. To prevent frothing, 1 to 4 liters of 3 per cent octadecanol in lard oil was automatically added as required. The 200 liters of inoculated medium in each tank was stirred at 270 rpm and aerated at 200 liters per minute. The tanks were kept under a pressure of 20 pounds per sq inch.

TABLE 6

Comparison of NaOH and $CaCO_3$ as neutralizing agents

CULTURE	NaOH				$CaCO_3$			
	Run no.	Penicillin yield	Age at maximum yield	pH at maximum yield	Run no.	Penicillin yield	Age at maximum yield	pH at maximum yield
		units/ml	*hr*			*units/ml*	*hr*	
R-38	74	72	67	8.3	75	100	92	7.4
R-38	79	93	70	7.3	78	135	55	7.8
1951-B25	81	153	78	7.3	80	174	62	7.4
25099	83	27	72	5.6	82	244	62	7.2
25099	87	85	87	7.4	86	187	68	7.5

The initial pH values of these fermentations were always between 5.6 and 6.1.

COMPARISON OF CULTURES

Because of the variations in yields caused by unknown and uncontrolled factors, it was necessary during the fermentations in which various cultures were compared to intersperse control runs on a known culture. *P. chrysogenum* 1951-B25 was used for this purpose, since it was the highest-yielding culture known at the time the tests were begun. Table 7 presents a summary of all fermentations run under the standard culture comparison conditions. It will be seen that several of the Stanford cultures were apparently slightly superior to the 1951-B25 control, but none of the isolations from natural sources surpassed this control culture. The ultraviolet mutant from Minnesota (*Penicillium* strain 15-U-1) appears similar to the Stanford mutant from the standpoint of penicillin yield. However, the X-ray mutant from the Carnegie Institution (*P. chrysogenum* X-1612), which was only slightly better than the other cultures in bottle fermentations, proved to be definitely superior to all the previously tested cultures under tank conditions. This culture was used for further testing of the tank conditions, but a variability and a loss of penicillin-producing activity took place over the course of the experiments. Table 8

TABLE 7

Summary of penicillin yields in culture comparison experiments

(Tank fermentation)

CULTURE	NO. OF FERMENTATIONS	PENICILLIN YIELD		CONCURRENT YIELD WITH 1951-B25*
		Average	Best	
		units/ml	*units/ml*	*units/ml*
832	1		98	214
1951-B25	7	169	245	
25099	2	216	244	184
35217	1		275	245
35347	1		255	245
45417	2	199	229	245
15-U-1	3	157	200	214
R-38	2	109	135	165
R-1139	2	158	160	214
R-1204	2	147	148	214
R-1205	2	160	191	214
X-1612	46	369	558	245
Q176	6	761	904	
Q176-A8	6	286	360	

* This culture was widely used in experimental and early industrial fermentations; hence, control fermentations were run at intervals in order to compare them more accurately with other cultures.

TABLE 8

Loss of activity of P. chrysogenum X-1612

SOIL STOCK	PERIOD OF FERMENTATIONS	NO. OF FERMENTATIONS	AVERAGE OF PENICILLIN YIELDS
			units/ml
I	Jan. through March, 1945	13	421
I	April through June, 1945	16	344
I	July through Sept., 1945	9	290
II	Sept. through Dec., 1945	8	423

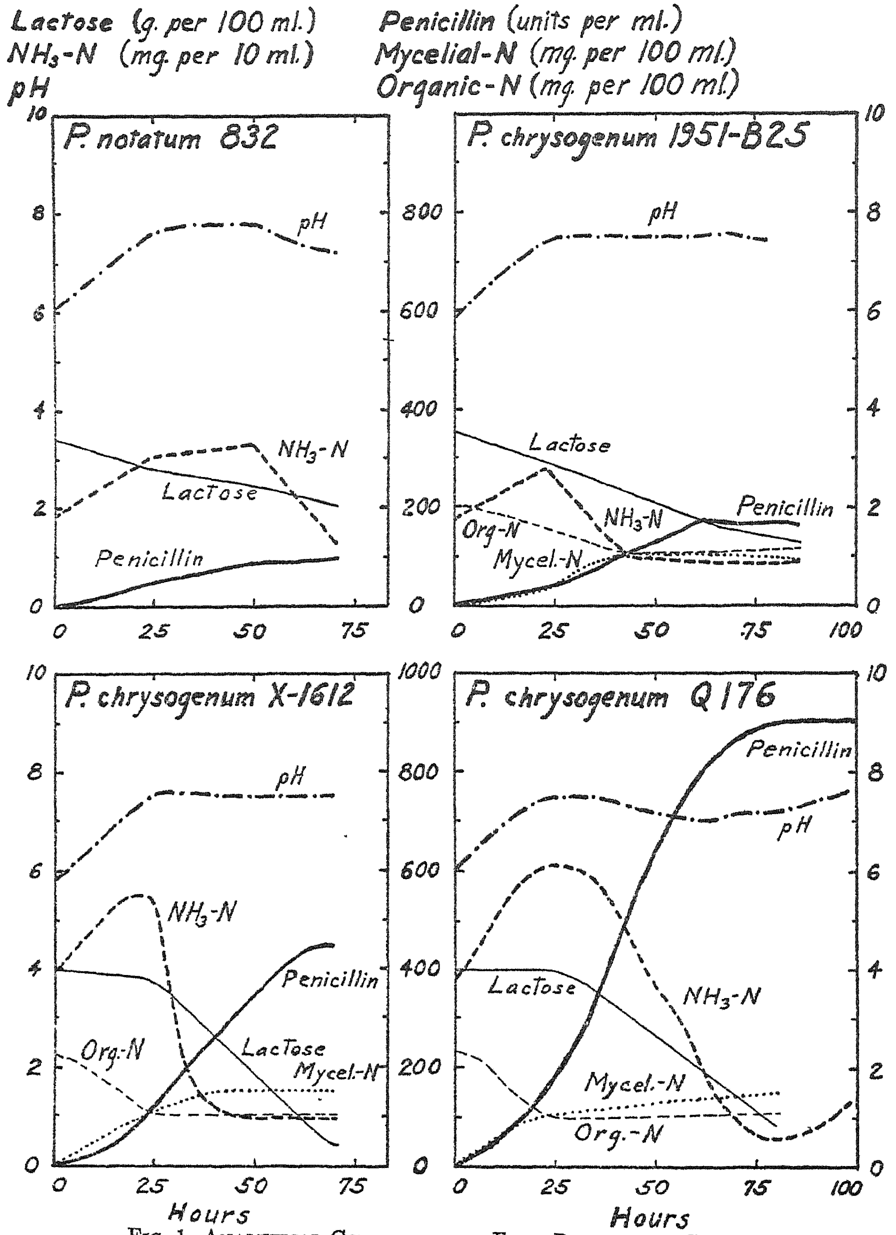

FIG. 1. ANALYTICAL COMPARISON OF FOUR PENICILLIUM STRAINS

Medium: 3 to 4 per cent lactose, 4 per cent steep liquor solids, and 1 per cent calcium carbonate.

shows a separation of the 46 standard fermentations by strain X-1612 into four groups, the first three groups representing the progressive aging of soil stock I (kept at room temperature). This loss of activity could not be correlated with any other factor, such as differences between lots of steep liquor. Soil stock II was somewhat closer to the original strain of X-1612 (it had been stored in a cool room). Fermentations with it appeared to be similar to the early fermentations with the other soil stock culture.

The ultraviolet mutant from strain X-1612, called Q176, and the single cell isolation from it, strain Q176-A8, gave promising results in shaken-flask experiments, appearing equivalent to each other but superior to the parent strain. The tank fermentations revealed the superiority of strain Q176, but its variability in fermentations indicates that it may be an unstable culture.

It will be seen from table 7 that only a few fermentations were run on the majority of the cultures. Hence, chemical analyses and penicillin values are not available for an accurate comparison of these cultures. Figure 1 is a graphical representation of the analyses of several typical fermentations. *P. notatum* NRRL832 is included for comparison, since it was used widely in early experiments. There is no assurance that this represents its normal behavior in tank fermentations. The other three graphs are more or less representative of the normal or average tank fermentations for these strains of *P. chrysogenum* under our standard conditions. Strain Q176 uses lactose more slowly than strain X-1612. The liberation of ammonia from organic nitrogen during mycelium formation is evident in these fermentations. When the available organic nitrogen is utilized, further growth occurs at the expense of lactose and ammonia nitrogen. Mycelial growth is limited by the amount of organic nitrogen and ammonia nitrogen in the medium (since an increase in mycelial nitrogen ceases upon the exhaustion of available ammonia nitrogen). Because strain Q176 grows more slowly than the strains 1951–B25 and X-1612, it uses ammonia nitrogen more slowly than the parent strains.

ACKNOWLEDGMENT

The authors are indebted to Dr. W. H. Peterson for counsel in this work, to R. W. Rivett and R. M. Dodson for conducting the bottle fermentations, to F. G. Jarvis for aid in conducting the tank fermentations, and to Margaret Larson for the penicillin assays.

SUMMARY

The penicillin-producing properties and the gross metabolic characterstics of a number of strains of the *Penicillium notatum-chrysogenum* group were compared. The fermentations were conducted in 100-gallon tanks.

The conditions, aside from adequate aeration, apparently necessary for optimum penicillin production are rapid initial production of mycelium, the presence of a slowly fermentable carbohydrate and available nitrogen (e.g., ammonia), and the maintenance of a pH value between 7.0 and 7.8.

The penicillin yield under these conditions varies widely with the culture used. Of the many strains tested, the mutant *P. chrysogenum* strains X-1612 and Q176 have been found to produce the highest yields. Strain X-1612 yields 400 to 500 units per ml, and strain Q176 gives 700 to 900 units per ml.

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NOTES

THE PREPARATION OF LOEFFLER'S SERUM AND SIMILAR COAGULABLE MEDIUMS

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Received for publication March 23, 1946

Foster and Cohn (J. Bact., **50**, 561) have recently presented a method for the preparation of certain coagulable mediums that appears to involve unnecessary complications. During the past ten years we have had unfailing success by the following procedure:

Eighty grams of Difco Loeffler's blood serum (dehydrated) are added to 1 liter of distilled water. This mixture is heated to 50 C in a double boiler or on a constant temperature electric hot plate for 45 minutes. During this time it is agitated and dissolved by an electric stirring apparatus run at such speed as to avoid unnecessary bubbling or frothing. By this procedure the powder is completely dissolved, and the slants, after sterilization, are creamy white with no muddy sediment.

After the stirring and solution the medium is tubed in 6-by-$\frac{5}{8}$-inch tubes, about 4 ml being added to each tube. During the tubing and plugging period the autoclave is heated to steaming.

The tubes of media are placed in wire baskets, about 12 tubes in 2 layers to each basket. If the steam enters the bottom of the autoclave, the baskets are placed in a slanting position on a cloth or cardboard on the floor of the autoclave at such an angle that the slant extends to the bottom of the tube, leaving no "butt" to cause blowing of the medium by engendered pressure. If the steam enters at the top, the slanted baskets are covered with a layer of cloth or cardboard.

The door and port of the preheated autoclave are closed, the steam (or heat) is turned up, and the pressure of air and steam is raised quickly to 15 pounds, at which it is held for 20 minutes at a temperature of about 80 to 85 C. After this period of coagulation the port is cautiously opened to release the air while the pressure of live steam is carefully held at 15 pounds. When all air is removed, as shown by the issue of live steam from the port, this is closed and the temperature is raised to 121 C and held again for 20 minutes for sterilization.

After 20 minutes the steam (or heat) is reduced slowly until the pressure approaches zero, when the port is carefully and gradually opened to avoid a negative pressure.

The same method of sterilization may be successfully applied to the preparation of Dorsett's, Petroff's, and similar coagulable mediums.

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THE ACTIVITY OF STREPTOMYCIN IN RELATION TO BACTERIAL SPORES AND THE PRESERVATION OF MILK

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Received for publication April 1, 1946

A study was completed recently of the activity of penicillin against bacterial spores in milk (in press). During the first part of this work some parallel observations were made also on streptomycin. When the very limited effectiveness of the latter agent became apparent, the work was discontinued. Since the literature contains no reference to the activity of streptomycin against bacterial spores, the essential findings are briefly recorded.

The organisms, their sources, and the methods and techniques employed were as described in the report concerning penicillin. The streptomycin was the commercial product (Merck) dissolved in sterile distilled water. After the seeding of tubes of sterile (autoclaved) milk with the washed spores, the inoculated samples were heated at 95 C for 15 minutes, cooled, and 5 u per ml of streptomycin were added. The number of viable spores before heating was approximately 50,000 per ml.

The milk inoculated with *Bacillus megatherium* (4 strains tested) and treated with streptomycin remained unchanged during 3 months of storage at 30 C. The controls with no streptomycin were visibly spoiled within 10 days.

Visibly spoiled within 10 days were both test and control samples of *Bacillus laterosporus* (2 strains), *Bacillus cereus* (5 strains), *Bacillus mycoides*, *Bacillus metiens*, *Bacillus subtilis* (11 strains), *Bacillus pumilus*, *Bacillus brevis* (3 strains), *Bacillus macerans*, *Bacillus polymyxa*, *Clostridium botulinum*, and strain 3679 (anaerobe). Both test and control samples of *Bacillus alvei* spoiled in about 1 month.

Later observation revealed that 100 u per ml of streptomycin were not sufficient to prevent spoilage of milk containing as few as 100 per ml of viable spores of *C. botulinum* and of 3679 (anaerobe).

On each of 3 days, raw whole milk receiving no artificial inoculation was heated at 95 C for 15 minutes, cooled, and treated with streptomycin at the rate of 50 and 100 u per ml. The count after heating varied from 1 to 46 per ml (spores). All samples containing streptomycin visibly spoiled within 1 month under both aerobic and anaerobic cultivation (30 C); control samples spoiled in 3 days.

The foregoing observations indicate that streptomycin in ordinary concentrations has very limited activity against bacterial spores.

HYDROGEN SULFIDE FORMATION BY SHIGELLA ALKALESCENS

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The *Shigella* are generally regarded as organisms that fail to form hydrogen sulfide. In a recent report on a lead semisolid agar, Friewer and Shaughnessy (Am. J. Clin. Path., **8**, 1) observed the formation of H_2S by several strains of *Shigella paradysenteriae* (Flexner) and *Shigella sonnei* in this medium and also by the lead acetate paper test, although no mention was made of this reaction by *Shigella alkalescens*. Dr. C. A. Stuart (personal communication) states that he has encountered no alkalescens cultures that produce H_2S.

During 1945 we isolated 232 strains of *Shigella alkalescens* in the central laboratory at Jacksonville. Of these, the production of H_2S in Kligler's iron agar was noted by 5 cultures. This reaction was not apparent after 24 hours' incubation and was only slight after 48 hours at 37 C, but a very distinct reaction appeared after another 24 hours at room temperature. The first 3 cultures were isolated from routine feces specimens from different persons between May 9 and May 21, 1945. We continued to watch all alkalescens cultures isolated very closely, although H_2S was not detected again until September 13, 1945, when it was noticed in a fourth culture. The fifth culture was isolated on October 9, 1945.

Since one freshly isolated strain of *S. alkalescens* has been found by Wheeler *et al.* (J. Bact., **51**, 169) to form acetylmethylcarbinol, others to ferment lactose and sucrose (Stuart and Rustigian: J. Bact., **48**, 497) and to lose these characteristics shortly afterwards, it was thought that the ability to produce H_2S might also be lost. In January, 1946, the cultures isolated in September and October were forwarded to Dr. Stuart, who found that the reaction, though somewhat weak, was very distinct and also that these cultures contained the antigens A, B, C, and D. One of the cultures found in May was lost. The remaining 2 were checked 7 months after isolation. One strain produced a very distinct reaction after 48 hours, and in 72 hours there was a diffuse blackening in the entire butt of the Kligler culture. The other strain showed no reaction in Kligler's medium, though it is possible the formation would be detected by more sensitive tests.

The observation that some strains of *Shigella alkalescens* form hydrogen sulfide in amounts equivalent to *Salmonella typhi* and apparently do not lose this characteristic seemed worthy of note.

The authors wish to acknowledge their appreciation for the advice and criticism received from Dr. C. A. Stuart and Dr. A. V. Hardy.

TRIMETHYLAMINE OXIDE REDUCTION AND THE EIJKMAN REACTION IN THE GENUS ERWINIA

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The success that has been attained in separating species of *Shigella* by means of the trimethylamine oxide reduction test and the Eijkman reaction has prompted this investigation. Past studies with the genus *Erwinia* bearing on trimethylamine oxide reduction (Wood and Baird: J. Fisheries Research Board Can., **6**, 194) and the Eijkman reaction (Stuart *et al.*: J. Bact., **43**, 557) have not indicated the species employed. In the former study it was found that the genera *Erwinia* and *Shigella* were the only ones in the family *Enterobacteriaceae* which contained species that did not have the ability to form trimethylamine from the oxide. It was revealed that 8 out of 17 cultures of the plant pathogens were able to reduce trimethylamine oxide. Applying the Eijkman test, Stuart *et al.* (*loc. cit.*) showed that, with 24 cultures of *Erwinia*, 20 failed to survive at 45.5 C for 24 hours. Later Wood *et al.* (J. Bact., **46**, 106) stated that in the genus *Shigella* a direct correlation existed between the Eijkman reaction and the ability to reduce trimethylamine oxide.

TABLE 1

Trimethylamine oxide reduction and the Eijkman reaction with species of Erwinia

ERWINIA SPECIES	NUMBER OF CULTURES	TRIMETHYLAMINE OXIDE REDUCTION		EIJKMAN REACTION*		
		Positive	Negative	O	G	A
E. amylovora	4	0	4	4	0	0
E. tracheiphila	3	0	3	3	0	0
E. phytophthora	4	0	4	4	0	0
E. solanisapra	3	0	3	3	0	0
E. carotovora	13	3	10	13	0	0
E. aroideae	15	4	11	15	0	0
E. carnegieana	1	1	0	0	0	1
E. nimipressuralis	1	1	0	0	1	0
Totals	44	9	35	42	1	1

* O, no growth and not viable; G, growth; A, acid.

Comparative studies pertaining to the capacity to reduce trimethylamine oxide and the Eijkman reaction in the genus *Erwinia* are presented herewith. Forty-four cultures of *Erwinia* representing 8 species have been utilized. The results are given in table 1. All available cultures of *Erwinia amylovora*, *E. tracheiphila*, *E. phytophthora*, and *E. solanisapra* failed to form trimethylamine. Out of 13 isolates of *E. carotovora*, 10 were unable to reduce trimethylamine oxide, but 3 had this capacity. With 15 cultures of *E. aroideae*, 11 were negative in their action on trimethylamine oxide, whereas 4 were able to produce trimethylamine. Single strains of *E. carnegieana* and *E. nimipressuralis* had the property of reducing trimethylamine oxide.

All of the *Erwinia* cultures, except *E. carnegieana* and *E. nimipressuralis*, failed to grow or remain viable under the conditions of the Eijkman test (glucose was substituted for lactose in the medium employed). These two Eijkman survivors, which were also trimethylamine-positive, are like *Shigella alkalescens*, *S. dispar*, and *S. sonnei* in respect to the Eijkman test and trimethylamine oxide reduction. With *E. carotovora* and *E. aroideae*, however, no correlation exists between the trimethylamine-positive strains and the ability to withstand the conditions of the Eijkman test.

No comparative studies have been made between trimethylamine-positive and trimethylamine-negative strains of the latter two species. Nevertheless, the faculty to macerate carrot tissue is maintained by negative and positive isolates of both species. The existence of strains in the soft-rot group that have the power of reducing trimethylamine oxide preclude the use of the test in distinguishing these species from members of the genus *Aerobacter*.

THE ANTIBIOTIC ACTIVITY OF VIOLACEIN, PRODIGIOSIN, AND PHTHIOCOL

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The water-insoluble pigments violacein, prodigiosin, and phthiocol are produced, respectively, by *Chromobacterium violaceum*, *Chromobacterium prodigiosum*, and *Mycobacterium tuberculosis*. The antibiotic activity of violacein has been reported (Lichstein and Van de Sand: J. Infectious Diseases, **76,** 47) but to our knowledge there have been no similar studies with phthiocol. Although whole cultures of *Chromobacterium prodigiosum* have long been recognized as possessing antibiotic activity, there has been a difference of opinion regarding the nature of the active substance. Hettche (Arch. Hyg. Bakt., **107,** 337) believes the pigment to be the active agent, but Eisler and Jacobsohn (Z. Hyg. Infektionskrankh., **117,** 76) suggest that the substance is a nonpigmented material present in broth cultures of the organism.

Violacein, extracted and purified according to the method of Strong (Science, **100,** 287), exerts a bactericidal action against *Staphylococcus aureus* in concentrations of 0.001 to 0.01 per cent when the number of bacterial cells is approximately 5,000 per ml, and a bacteriostatic action against larger inocula. The pigment exhibits no *in vitro* antiphagocytic activity against guinea pig exudative polymorphonuclear leucocytes in concentrations of 0.05 to 0.5 mg. Mice (8.5

to 16 g) tolerate a single intraperitoneal dose of 1 to 2 mg of pigment. No *in vivo* protection against overwhelming type II pneumococcus infection in mice is exhibited by single doses of 1 to 2 mg, but a delayed death rate is noted with 2 mg against smaller challenge doses of organisms (10^{-5} dilution).

Prodigiosin, extracted and purified from potato slice cultures (Wrede and Hettche: Ber. deut. chem. Ges., **62**, 2678) and tested against 10 representative species of bacteria in tryptose broth using an inoculum of 10^4 organisms, with suitable alcohol controls, exhibited no bacteriostatic effect in concentrations of 0.005 to 0.1 per cent. Both filtrates and heat-killed 18-hour and 3-week whole cultures of *Chromobacterium prodigiosum* were employed in order to test the effect of other metabolic products. Young cultures exhibited no activity, but the 3-week cultures, both pigmented and nonpigmented, yield a thermostable, water-soluble, nonpigmented substance that is antagonistic to the growth of *Bacillus subtilis*, *Corynebacterium diphtheriae*, and *Staphylococcus aureus*.

The stock solution of phthiocol was 2 per cent in 0.2 N sodium hydroxide, and, when added to tryptose broth, an equal volume of 0.2 N hydrochloric acid was required to maintain a suitable pH. In a concentration of 0.02 per cent this pigment inhibited the growth of *Streptococcus pyogenes*, *Diplococcus pneumoniae*, and *Bacillus anthracis*, whereas 0.05 per cent inhibited the growth of these organisms and moreover that of *Staphylococcus aureus*, *Streptococcus salivarius*, *Corynebacterium diphtheriae*, *Escherichia coli*, and *Shigella paradysenteriae*. A concentration of 0.1 per cent was bacteriostatic for *Eberthella typhosa* and *Neisseria catarrhalis*, but was not sufficient to inhibit the growth of *Pseudomonas aeruginosa*.

THE OCCURRENCE OF SALMONELLA BLEGDAM IN THE PHILIPPINES

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Of 19 strains of *Salmonella* isolated during the first year of the U. S. reoccupation of the Philippines 4 proved upon full investigation to be *S. blegdam*. This organism was originally described by Kauffmann in 1935—a single isolation from the blood of a middle-aged patient in the Blegdam Hospital, Copenhagen, suffering from pneumonia of the right lower lobe. First given a varietal status, this species differs serologically from *Salmonella enteritidis* primarily in its possession of the "q" flagellar antigen in addition to the "g" and "m"; and from *S. enteritidis* var. *moskow* in its possession of the "m" factor.

Two of the four strains were isolated from the blood of American infantry soldiers showing distinct symptoms of paratyphoid infection; one from the stool of a soldier without apparent enteric fever; and the last from an ulcerative ankle lesion on a Filipino patient, also asymptomatic with respect to gastro-intestinal involvement.

Credit for the final identification of these cultures is due Dr. P. R. Edwards, of the University of Kentucky Agricultural Experiment Station, and to Miss Alice Moran, of his staff.

A NOTE ON SALMONELLA ABORTUS-EQUI INFECTION IN MAN

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Received for publication April 15, 1946

Although many of the delineated *Salmonella* types frequently appear in certain infections in man, the occurrence of *Salmonella abortus-equi* in human diseases rarely has been reported. Fujimura and Hoshi (Japan. Soc. Vet. Sci., **1936**, 159) reported the case of a veterinarian in Japan who developed skin abscesses of his arm after treating the genital tract of a mare which had aborted because of *S. abortus-equi* infection. *S. abortus-equi* and a *Staphylococcus* were isolated from the purulent exudate of the infected lesions. Bornstein, Saphra, and Strauss (J. Infectious Diseases, **69**, 59) describe a culture of *S. abortus-equi* that was received from Professor Curbelo of Havana, Cuba. It was listed as an isolation from the feces of a human patient who probably became infected in the laboratory.

During the month of August, 1945, Lieutenant Colonel Rushmore, Veterinarian, Allied Military Government, Italy, sent several *Salmonella* cultures to the 15th Medical General Laboratory for serological classification. Among the cultures, two were listed as isolations from a food-poisoning outbreak at Bresca, Italy. One of these cultures was isolated from horse meat suspected of causing the outbreak, and the other was obtained from the feces of a case of gastroenteritis in an Italian civilian who had eaten some of the meat. A serological investigation of the antigens of both cultures according to the Kauffmann-White schema proved them to be *S. abortus-equi* (IV, XII:–e, n, x . . .).

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MISSOURI VALLEY BRANCH

CITY AUDITORIUM, TOPEKA, KANSAS, NOVEMBER 30 AND DECEMBER 1, 1945

AGGLUTININ RESPONSES TO THE O AND OH ANTIGEN OF TYPHOID VACCINE ADMINISTERED ORALLY TO RABBITS. *W. Howard Baker and J. Ralph Wells*, Kansas State Teachers College, Pittsburg, Kansas.

A comparative study was made of the agglutinin response of bile-prepared rabbits, following oral administration of the OH antigens of typhoid vaccine to one group and the O antigen of the same vaccine to another group, when administered daily and twice daily. The results showed that all animals possessed agglutinin titers ranging from 1:10 to 1:320 on the eighth day after the administration of the last dose. There was not enough difference in the titers resulting from the use of the antigens to say safely that the OH antigens produced a higher titer than the O antigen or vice versa. Apparently the agglutinin titers for OH persisted longer than did those resulting from the use of the O antigen. The results obtained by the two frequencies were essentially the same with respect to both the height and the persistence of the titer.

SOME FUNGI INVOLVED IN THE DECOMPOSITION OF PLANT RESIDUES. *R. C. Dawson*, U. S. Department of Agriculture, Soil Conservation Service and Research, Nebraska Agricultural Experiment Station.

Representative types of fungi were isolated from the soil of plots treated with different kinds and placements of crop residues and from fragments of decomposing residues. Members of the genera *Penicillium* and *Aspergillus* were most commonly found in the soil. *Chaetomium* was frequently found in large numbers on straw and cornstalks during intermediate stages of decomposition. Other genera encountered were *Alternaria*, *Rhizopus*, *Hormodendrum*, *Trichoderma*, *Fusarium*, and two the identification of which are at present unknown.

All of the organisms were tested quantitatively for their ability to decompose wheat straw and for their ability to utilize cellulose and lignocellulose. The decomposition of a legume (alfalfa) by some of the organisms was also studied. Carbon dioxide and ammonia evolution, loss in weight, and changes in pH were used as criteria of decomposition. Different fungi varied considerably in the rate and type of decomposition produced.

A COMPARISON OF VARIOUS EGG MEDIA FOR THE ROUTINE ISOLATION OF MYCOBACTERIUM TUBERCULOSIS. *Eleanore Herndon and Charles A. Hunter*, Kansas State Public Health Laboratories.

Employed in this study were five media for the cultivation of *Mycobacterium tuberculosis*, including two non-glycerol-containing media for the cultivation of the bovine type. The latter were Sweany's medium and Arena and Cetrangolo's modification of Sweany's medium. Corper's egg yolk medium, Petrik's modification of Loewenstein's medium, and Saenz' modification of Petragnani's medium were used for cultivation of the human type. It was found that the use of no single medium would give satisfactory results, and that it is advisable to use three media. The combination found most satisfactory in this study included Saenz', Petrik's, and Arena and Cetrangolo's media. Reasons for this choice were as follows: (1) Saenz' gave a higher percentage of positives than did the other two media suitable for the cultivation of the human type, (2) Petrik's gave a few positives when the other two did not, (3) Corper's medium was found to be very liable to contamination and in no case gave a positive when the other two were negative, and (4) Arena and Cetrangolo's medium gave a higher percentage of positives than did Sweany's. Although growth of *M. tuberculosis* appeared most frequently on the Arena-Cetrangolo medium when all other tubes were negative, the colonies were frequently atypical in appearance. This was probably due to the fact that they were colonies of the human type, growing in the absence of glycerol.

The use of a shaking machine during digestion of the specimens was found to decrease contamination of cultures, and maximum and even seeding was effected. The percentage of microscopically negative specimens found to be positive by culture was increased from 6.4 to 10.4 per cent with the use of shaking plus the use of the Arena-Cetrangolo medium.

THE INFLUENCE OF SOME MICROBIAL GROUPS ON SOIL STRUCTURE STABILITY. *T. M. McCalla*, U. S. Department of Agriculture, Soil Conservation Service and Research, Nebraska Agricultural Experiment Station.

A laboratory study was made of the effect of different microbial groups on soil structure stability. Lumps of Peorian loess were used for a testing material. The stability developed was determined by the number of water drops required to destroy lumps of the treated material. Energy material was added in the form of carbohydrates, proteins, wheat straw, and sweet clover residues. These were inoculated and incubated for different lengths of time.

The order of decreasing stability was fungi, actinomycetes, yeast, and bacteria. The fungi and actinomycetes were highly effective in soil stabilization. There were wide differences among the different bacteria. Some were highly effective, but others were without much influence.

These results would indicate that cultural practices encouraging the development of fungi, actinomycetes, and certain bacteria would be effective in improving soil stabilization.

MODE OF ACTION OF MARFANIL. *Dan Tenenberg*, Department of Bacteriology, University of Kansas, Lawrence.

THE USE OF SELECTIVE ENRICHMENT MEDIA FOR THE ISOLATION OF ENTEROCOCCI. *L. F. Lindgren*, Department of Bacteriology, University of Nebraska, Lincoln.

GEOGRAPHICAL DISTRIBUTION OF HISTOPLASMIN SENSITIVITY. *M. L. Furcolow*, Tuberculosis Control Division, U.S.P.H.S., Hixon Memorial Laboratory, University of Kansas Hospital, Kansas City, Kansas.

THE INHIBITION OF BACTERIAL RESPIRATION BY CRYSTAL VIOLET AND ITS RELATIONSHIP TO GRAM'S STAIN THEORY. *John Harris*, Department of Bacteriology, Kansas State College, Manhattan.

EXPERIMENTAL STUDIES IN LEPTOSPIROSIS. I. PRELIMINARY REPORT ON THE EXPERIMENTAL THERAPY OF LEPTOSPIROSIS. *B. S. Levine and Charles A. Hunter*, Division of Public Health Laboratories, Kansas State Board of Health, Topeka.

WESTERN EQUINE ENCEPHALITIS. *C. M. Eklund*, Tuberculosis Control Division, U.S.P.H.S., Hixon Memorial Laboratory, University of Kansas Hospital, Kansas City, Kansas.

SURVIVAL OF PASTEURELLA TULARENSIS IN WATER. *Cora M. Downs*, Department of Bacteriology, University of Kansas, Lawrence.

FURTHER STUDIES ON LISTERELLOSIS IN RABBITS. *Barbara Russell and N. P. Sherwood*, Department of Bacteriology, University of Kansas, Lawrence.

VITAMIN A IN MASTITIS. *V. D. Foltz*, Department of Bacteriology, Kansas State College, Manhattan.

THE ACCUMULATIVE EFFECT OF INDOLE AND BILE UPON EBERTHELLA TYPHOSA AND THE SHIGA DYSENTERY ORGANISM. *H. L. Chance*, Department of Bacteriology, University of Oklahoma, Norman, Oklahoma.

EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND EIGHTY-FIFTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, PHILADELPHIA, PENNSYLVANIA, FEBRUARY 26, 1946

INTERFERENCE WITH THE ANTIBACTERIAL ACTION OF STREPTOMYCIN BY REDUCING AGENTS. *Amedeo Bondi, Jr., Catherine C. Dietz, and Earle H. Spaulding*, Department of Bacteriology, Temple University School of Medicine, Philadelphia 60, Pa.

The antibacterial activity of streptomycin in infusion agar plate cultures of *Escherichia coli* and other bacteria is diminished by anaerobic incubation. The bacteriostatic activity of this antibiotic for *E. coli* is reduced in culture broth containing cysteine and sodium thioglycolate. That this interference by these sulfhydryl compounds is related to their reducing powers is supported by the fact that various inorganic reducing agents likewise interfere with the action of streptomycin. Stannous chloride, sodium bisulfite, sodium hydrosulfite, sodium formate, and sodium thiosulfate definitely antagonized the antibacterial action of this antibiotic. The concentration required for bacteriostasis was frequently increased as much as 16- to 64-fold by the presence of some of the organic and inorganic reducing agents. The possible relationship between this phenomenon and the mode of action of streptomycin was discussed.

CLINICAL BACTERIOLOGY AND IMMUNOLOGY OF OCULAR INFECTION. *Charles Weiss*, Jewish Hospital, Philadelphia, Pa.

MICHIGAN BRANCH

DETROIT, MICHIGAN, MARCH 29, 1946

A NEW POSITIVE GERMICIDAL TREATMENT FOR STERILIZING JUTE-PACKED WATER MAINS. *Alfred L. Sotier*, Research Department, Wyandotte Chemicals Corporation.

An experimental product known as polymine D, containing 5 per cent of quaternary ammonium germicide combined in solid form with mild alkalies which enhance its germicidal power, has proved to be effective for sterilizing two jute-packed water mains that had proved refractive to chlorine sterilization.

Considerable time and effort were expended on the shorter main (300 feet) in learning to apply the germicidal solution for best results—how long to allow the solution to contact the pipe and its packing and what strength of solution to use. From the knowledge gained on this short main, it was possible to sterilize the longer main (3,000 feet) with one 7-day application of germicidal solution.

The relatively rapid and sure action of this formulation for this particular use can be attributed both to its high germicidal power and to its detergent and surface-active properties, which enable it to wet out and penetrate quickly and effectively all of the fibers of the packing.

THE VALUE OF SEROLOGIC TESTS IN TRICHINOSIS. *Arthur Frisch*, Wayne University Medical School, Detroit.

STREPTOBACILLUS MONILIFORMIS INFECTION WITHOUT A RECENT HISTORY OF A RAT BITE. *Carl E. Duffy and Lucille F. Roberts*, Wayne University Medical School and Receiving Hospital, Detroit.

NEW YORK CITY BRANCH

NEW YORK, APRIL 2, 1946

FACTORS AFFECTING THE ACCURACY OF COAGULASE TESTS OF STAPHYLOCOCCI. *George H. Chapman*, Clinical Research Laboratory, New York.

Different factors involved in the coagulation of blood by staphylococci are discussed, and reasons are presented for the development of the following technique, which gives positive results in one-half to one hour, with additional advantage that a single culture medium is used for isolation, coagulase, mannitol fermentation, chromogenesis (with agreement of the three tests in 95 per cent of the cultures), and the Stone reaction. A standard strain, grown on the special medium (see abstract of meeting, Dec. 27, 1945) is used to determine the clotting time of blood samples. Only those clotting rapidly are used in the tests. The method for testing unknown cultures is as follows: Mix a loopful of the culture, grown exactly 42 hours on the special medium, with 0.1 ml of bacto brain heart infusion, shake violently at frequent intervals for exactly 15 minutes, and add 0.2 ml of acceptable tested blood. Incubate the mixture, preferably in a water bath, at 37 C, and inspect in exactly one hour. Use as a control a strain that has extremely feeble clotting power.

ESSENTIAL ELEMENTS FOR PURPLE BACTERIA OCCURRING AS IMPURITIES IN CP IRON AND MANGANESE. *S. H. Hutner*, Haskins Laboratories, New York.

TRIAL OF VOLATILE COMPOUNDS AS REMOVABLE ANTISEPTICS. *Clara A. Bjerknes and S. H. Hutner*, Haskins Laboratories, New York.

To prevent microbial growth in dilute, acidified solutions of cp salts of boron, zinc, and molybdenum and other compounds, inorganic and organic, that commonly contain assortments of trace elements favorable to growth, and also to kill cultures of actinomycetes for assay of the antibiotic effect of their sterile metabolism fluids, an attempt was made to find an effective antiseptic removed by autoclaving or by the heat involved in pouring agar plates. In addition to adequate toxicity, the ideal compound should have a specific gravity near 1.0 for the sake of remaining evenly distributed in diluted aqueous solutions after shaking; should boil below 121 C; should be immiscible with water to ensure removal by the steam distillation effect; and should be chemically stable.

Sixteen compounds, mostly alkyl halides, were tested for volatility, preservation of unsterilized broth media, and killing of young cultures of an actinomycete, of 10 other bacteria representing some of the commonly occurring contaminants, and of a torula.

Chloroform, ether, and *t*-butyl chloride, though effective antiseptics, were not sufficiently stable. At low concentrations (1 and 2 per cent), ethyl bromide, tetrachlorethylene, *n*-butyl chloride, and carbon tetrachloride proved most effective, but were not satisfactory in killing actinomycetes. From these studies it appeared that a fairly satisfactory preservative could be made by blending lighter-than-water *n*-butyl chloride with much heavier tetrachlorethylene or carbon tetrachloride.

THE CELLULAR TRANSFER OF TUBERCULIN HYPERSENSITIVITY. *Merrill W. Chase*, Rockefeller Institute for Medical Research, New York.

MUTATIONS TO SULFONAMIDE RESISTANCE IN STAPHYLOCOCCUS. *Eugene Oakberg and S. E. Luria*, Carnegie Institution, Cold Spring Harbor, New York.

Attempts to detect the presence of sulfonamide-resistant organisms in susceptible cultures of *Staphylococcus aureus* (strain NRRL313) by plating directly in sodium sulfathiazole (NaST) agar were hindered by inoculum size effect. It was found that this effect could be accounted for quantitatively by the production of antagonists during the early noninhibited phase of bacterial growth. By the use of bacteria inhibited by previous growth in the presence of NaST, it was possible to detect a few organisms with permanently increased resistance. Their frequency distribution showed the type of fluctuations that would have been expected if they had originated by mutation and were selected by the drug. The mutation rate was estimated at about 10^{-9} per bacterium per generation. The resistant bacteria in turn give mutants of higher resistance with comparable frequency. By successive additive mutations, 1,000-fold increases in NaST tolerance can be obtained. It was proved experimentally that the same mechanism accounted for the origin of highly resistant strains isolated by successive transfers in increasing amounts of NaST. Some of the resistant bacteria show increased production of an antagonist that can replace *p*-aminobenzoic acid as a growth factor for *Acetobacter suboxydans*, but the increased production of antagonist does not quantitatively account for the increased resistance of these strains. The relation between successive mutations and the synthesis of the growth factor is being investigated.

STUDIES IN STREPTOCOCCUS FAECALIS FOOD POISONING. *A. G. Osler, L. Buchbinder, and G. I. Steffen*, Bureau of Laboratories, New York City Department of Health.

THE SEROLOGY OF THE COLON GROUP. *F. Kauffmann*, State Serum Institute, Copenhagen, Denmark.

NORTHWEST BRANCH

UNIVERSITY OF WASHINGTON, SEATTLE, WASHINGTON, APRIL 3, 1946

INVESTIGATIONS OF THE GRAM-POSITIVE ANAEROBIC MICROCOCCI. *Edw. L. Foubert, Jr., and H. C. Douglas*, Department of Microbiology, University of Washington School of Medicine, Seattle 5, Washington.

Seventeen isolates of gram-positive, anaerobic, mass-forming, and tetrad-forming cocci have been made from the skin and blood plasma. All strains are nonpigmented, catalase-positive, coagulase-negative, nonhemolytic, and grow only under strictly anaerobic conditions. None of the isolates can be identified with previously described species.

Four strains which consistently form tetrads in all media have been provisionally identified as members of the genus *Gaffkya*. None of these strains ferment sugars; two of them form visible gas from peptone.

Thirteen isolates which are mass-formers have been provisionally placed in the genus *Micrococcus*. All strains ferment glucose, fructose, mannose, sucrose, and glycerol, with the formation of acid but no visible gas. Gas is not formed from peptones. Quantitative experiments on the nature of the sugar fermentation by one strain indicate that the principal dissimilation products are ethanol and CO_2 in equimolar amounts, formic and acetic acids in equimolar amounts, and small amounts of lactic acid.

The authors would appreciate receiving additional strains of anaerobic micrococci from other workers.

THE INCIDENCE OF STAPHYLOCOCCAL MASTITIS IN THE NORTHWEST. *Ernest C. McCulloch*, The State College of Washington, Pullman, Washington.

A survey of over 3,000 cows in Washington revealed 34 per cent to have one or more quarters showing some degree of abnormality. Of the incubated milk smears examined and quarter samples cultured, 64 per cent contained staphylococci and 28 per cent streptococci, the remainder being contaminants or miscellaneous types of infection.

The pathogenicity of staphylococci was checked by streaking on 5 per cent bovine blood agar, and after 24 hours' incubation at 37 C by streaking the hemolytic colonies in Difco phenol red mannitol agar to which was added an additional 70 grams of NaCl per liter. Staphylococci capable of hemolyzing bovine blood, growing on 7.5 per cent salt agar, and utilizing mannitol have been considered as pathogenic.

The infusion, immediately following milking, of 25,000 to 50,000 units of penicillin into the teat canal of quarters shedding staphylococci was followed by a temporary inability to culture staphylococci from the milk, but even the infusion of 100,000 units, repeated four times after four successive milkings, failed in several quarters to prevent the reappearance, after 7 to 14 days, of staphylococci in the milk.

The plate counts of penicillin-treated quarters show a tendency to approach gradually the levels of staphylococci found previous to treatment.

THE ISOLATION OF ERYSIPELOTHRIX RHUSIOPATHIAE FROM THE COMMON RAT. *Charles H. Drake and Elizabeth Hall*, Department of Bacteriology and Public Health, State College of Washington, Pullman, Washington.

OBSERVATIONS ON MENINGOCOCCUS CARRIERS. *B. S. Henry*, Department of Microbiology, University of Washington School of Medicine, Seattle 5, Washington.

THE NATURE OF THE CELLULOSE FERMENTATION IN THE RUMEN AND IN SLUDGE. *R. E. Hungate*, Department of Bacteriology and Public Health, State College of Washington, Pullman, Washington.

THE ACTION OF LEUCONOSTOC DEXTRANICUM AND LEUCONOSTOC CITROVORUM DURING THE RIPENING PROCESS OF AMERICAN CHEDDAR CHEESE. *Charles C. Prouty*, Department of Bacteriology and Public Health, State College of Washington, Pullman, Washington.

ERRATUM

On page 765 of the June, 1946, issue of the *Journal of Bacteriology*, ninth line from the bottom of the page, the number of grams reads "9.25." It should read "0.25."

ANTIMALARIAL AND ANTIBACTERIAL SUBSTANCES SEPARATED FROM HIGHER PLANTS[1]

H. J. CARLSON,[2, 3] H. D. BISSELL,[4] AND M. G. MUELLER[4]

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With the advent of penicillin many workers sought similar agents that were bacteriostatic or bactericidal *in vivo*. Such work has stimulated some investigators to seek like substances in higher plants. Osborn (1943) and Lucas and Lewis (1944) prepared extracts from a large series of plants and found many to contain inhibitory substances which were active against microorganisms *in vitro*. The object of the present study was to determine whether crude and refined extracts prepared from wild and domestic higher plants would inhibit the growth of microorganisms such as malaria parasites, nonpathogenic protozoa, bacteria, fungi, and viruses *in vitro* and *in vivo*.

In an attempt to separate inhibitory substances for malaria parasites, bacteria, and viruses, extracts were prepared from more than 200 wild plants collected in the semiarid region of southeastern Oregon and evaluated. Many of these have been found to contain certain substances which have proved to be bacteriostatic or bactericidal to microorganisms *in vitro*. The work was done without previous knowledge of work which has been published during the last two years.

A few of the genera studied are found throughout the world, such as *Artemisia* (sagebrush) and *Ranunculus* (buttercup). These two plants show a preference for the temperate and cooler regions of the northern hemisphere. Boas (1934) was the first to observe that extracts from species of *Ranunculus* retarded the growth of microorganisms. Further work by Boas and Steude (1935), Keding (1939), Schmidt (1942), Osborn (1943), and Seegal and Holden (1945) has supplied additional evidence that extractions from this family contain bacteriostatic and bactericidal properties against microorganisms. The available literature failed to reveal additional evidence on inhibitory activity of any of the other plants to be reported.

In vivo tests of each plant extract against malarial and bacterial infection would have been time-consuming and expensive without obtaining the desired knowledge. It was therefore decided to screen each plant extract by testing it on the seeded agar plate. If a specific plant extract was found to inhibit the test organism, it was then investigated further by more comprehensive *in vitro* and *in vivo* tests.

The plants to be reported on are buttercup (*Ranunculus occidentalis*, Nutt.), sagebrush (*Artemisia tridentata*), mountain pasque (*Anemone occidentalis*), dwarf waterleaf (*Hydrophyllum capitatum*), and juniper (*Juniperus occidentalis*, Nutt.).[5]

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[1] The opinions and views set forth in this article are those of the authors and are not to be considered as reflecting the policies of the Navy Department.

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Preparation of extracts. Fresh plants were finely ground with refined silica and a mortar and pestle, and then an equal volume of normal saline was added. This crude mixture was strained through two layers of gauze to remove larger particles, the filtrate being placed in the cold room until used. These suspensions will be referred to as crude saline extracts in the text and will be designated by the number C10 following the plant number, such as, buttercup extract P16C10.

TABLE 1

Plant preparations

Buttercup	
P16C10	Saline extract
P16B69	Volatile oil
P16B90	Na salt
Sagebrush	
P13C10	Saline extract
P13C10a	Saline extract concentrate
P13B24	Acetone-insoluble fraction
P13B31	Acetone-soluble gum
Dwarf Waterleaf	
P24C10	Saline extract
P24C10a	Saline extract concentrate
P24B1	Alcoholic extract
Mountain Pasque	
P158C10	Saline extract
P158B1	Steam distillate
P158B2	Volatile oil
P158B3	Ether-insoluble fraction of P158B1
Juniper	
P14C10	Saline extract
P14B1	Alcoholic extract
P14B2	Alcohol-insoluble, water-soluble fraction

Extracts with extreme pH levels were adjusted to 7.0. In the preparation of the refined extracts the following methods were followed in each case (table 1):

Buttercup extract P16B69. The extract was an oil obtained from ether extraction of the sodium-chloride-saturated steam distillate of the plant. The oil obtained was diluted 1:10 in absolute alcohol, and this was added to saline to obtain a final concentration of 1:400.

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[5] We wish to acknowledge our indebtedness to Dr. Helen M. Gilkey, Associate Professor, Curator of Herbarium, Department of Botany, Oregon State College, and Dr. Ira L. Wiggins, Professor of Biology, Stanford University, for their assistance in the identification of the plants.

Buttercup extract P16B90. The same procedure was followed through the ether extraction of P16B69. The ether extract was vigorously shaken with anhydrous Na_2SO_4 until the ether was dry. Metallic sodium was added to the dry ether. A brown precipitate was obtained. This precipitate was washed eight times with ethyl alcohol. The resulting substance was water-soluble and was designated as P16B90.

Sagebrush extract P13B24. The green portions of the plant were chopped and placed in diethylene dioxide. This mixture was incubated at 56 C for 3 to 4 days. The plant residue was filtered off, and the pungent dark green solution was concentrated *in vacuo* to a thick aromatic syrup. This syrup was boiled with 20 volumes of water for 5 minutes and filtered while hot. The chlorophyll was removed in the form of a resinous gum, which remained on the filter paper. The filtrate, tan in color and colloidal in appearance, was concentrated *in vacuo* again to a thick syrup or until foaming ceased. This residue was frozen with CO_2 ice, removed from the tube, and exposed to air for 12 to 18 hours. The brown gum obtained was extracted with acetone. This extraction yielded a yellow precipitate which was washed four times with acetone to remove the last traces of gum. This precipitate was water-soluble and designated as P13B24.

Sagebrush extract P13B31. The acetone-soluble fraction from the preceding extraction was evaporated to dryness *in vacuo.* The residue was a thick, aromatic, water-insoluble gum. This extract was made by dissolving the gum in alcohol and dispersing this solution in water.

Juniper extract P14B1. The berries of this plant were extracted with alcohol for 24 hours, and the berries were removed by filtration. The filtrate was evaporated to dryness. The residue was boiled with water, and the resulting colloidal suspension was designated as P14B1. Extract P14B2 was made by boiling the berries with water after the alcoholic extraction.

Waterleaf extract P24B1. The plant was soaked in normal saline solution for 24 hours at 8 C, filtered, and the residue extracted with alcohol for 30 days. The first 7 days the temperature was maintained at 37 C and thereafter at 8 C. The alcoholic extract was filtered and evaporated to dryness; the resulting residue was suspended in water and designated as P24B1. The plant concentrate was prepared by evaporating the saline suspension *in vacuo* at 45 C. The solution was concentrated to approximately 0.1 volume, and is known as P24C10a.

Mountain pasque extract P158B1. This extract was prepared by steam distillation of the plant. Ether extraction of the distillate and evaporation of the extract leave an acrid oil, which was designated P158B2. P158B3 was the aqueous solution after ether extraction.

In vitro tests. In the screening test used for each plant extract the Oxford cup method was used. One ml of an 18-hour culture of a recently isolated *Staphylococcus aureus* (hemolytic) was used to seed 100 ml of nutrient agar. After the shake cultures were added to the petri dishes, the agar surface was allowed to dry for 30 minutes. Sterile porcelain cylinders were placed on the surface of the seeded agar and filled with plant extracts. The plates were incubated at 37 C for 24 hours. Zones of partial or complete inhibition of growth of the organisms were measured in cm. When comprehensive tests were indicated, the same procedure was followed using different microorganisms. They included the molds, *Penicillium oxalicum*, *Penicillium notatum*, *Aspergillus* sp., and *Mucor* sp.; bacteria, *Corynebacterium diphtheriae*, *Pseudomonas aeruginosa*, *Eberthella typhosa*, *Clostridium sporogenes*, and *Streptococcus viridans;* and soil organisms, *Micromonospora* (Waksman), *Micromonospora* sp., and five species of *Streptomyces*.

Vapors from the various extracts were tested for their inhibitory activity by placing six drops of the extracts in the top of an inverted seeded agar plate prepared as described above. The cultures were incubated at 37 C for 24 hours. Zones of inhibition of growth were measured in cm. Activity for greater distances was obtained by placing a seeded agar plate over the top of a glass cylinder in which extracts were placed at the bottom. To remove any possibility that the liquids were being transferred by capillary action, a seeded plate was suspended in a bell jar having a diameter of 12 inches. The jar was incubated, and the inhibition zones were measured in 24 hours.

Dilution series were prepared of several extracts to ascertain their bacteriostatic and bactericidal characteristics. Varying amounts (1:1 to 1:2 × 10^4) of the extracts were added to nutrient broth, which was then seeded with the test organisms. The tubes were incubated at 37 C for 4 and 24 hours. Samples were taken after incubation and streaked on blood agar plates.

In vitro action of plant extracts against protozoa was determined by placing the organisms in the presence of the plant extract. Malaria-parasitized blood (*Plasmodium gallinaceum*) was mixed with extracts 1:1 and incubated at room temperature (25 to 29 C) for 6 hours. The blood was so diluted that it contained 8 × 10^6 parasitized cells per ml. One million of the treated cells were inoculated intravenously in 2-week-old chicks. These chickens were observed for 4 weeks, with blood smears being taken every other day.

Clone cultures of fresh-water protozoa (*Paramecium multinucleatum*, *Tetrahymena gellei*, and *Euglena* sp.) were added to 10 tubes, 1 ml each. To the first tube 1 ml of the saline extract of buttercup was added, 0.9 ml to the second tube, 0.8 to the third tube, and so on to the tenth tube to which 0.1 ml of the extract was added. The tubes were observed every 10 minutes for 1 hour.

Toxicity tests. Chickens: Four 2-week-old chicks were given 0.5 and 1 ml of the plant extracts by the intraperitoneal, intravenous, or subcutaneous route. The birds were observed 10 and 60 minutes and 24 hours after receiving the extracts. *Mouse:* Four 4-week-old white Swiss mice were inoculated with 0.25, 0.5, and 1.0 ml intraperitoneally and 0.5 ml subcutaneously of the plant extracts. The animals were observed at 10 and 60 minutes and at 24 hours after injection.

Chronic toxicity reactions were observed in those animals on test during prolonged treatment periods.

In vivo tests. The inhibitory action of the plant extracts on the malaria parasite was tried in the chicken. Three 2-week-old healthy chicks were injected intraperitoneally or subcutaneously with the extracts, 0.5 ml twice daily. The extract was started 2 days before infection with the malaria parasite. The infective dose of *Plasmodium gallinaceum* was 10^6 parasitized cells. Blood smears were taken every other day after the fourth to fifth day of inoculation. Birds succumbing after the ninth day of the disease were also observed for exoerythrocytic forms of the parasite in brain smears.

White Swiss mice, 4 weeks old, were inoculated intraperitoneally with 0.5 ml of a 6-hour broth culture of *Diplococcus pneumoniae* (type 19). One hour after inoculation with the test organism the mice were given intraperitoneal injections of the plant extracts. Thereafter the extract was given twice daily in predetermined doses. Animals dying in the test and control groups were autopsied and cultures taken. The degree of infection was determined by comparison with the untreated control infections.

TABLE 2

Effect of extracts of plants on Staphylococcus aureus by the Oxford cup method

Plant extracts	Inhibition-diffusion zones in cm (diameter)
Buttercup	
P16C10	3.5–9.0
P16B69	5.0*–9.0
P16B90	2.3
Sagebrush	
P13C10	1.8
P13B24	2.0
P13B31	1.3
Juniper	
P14C10	3.2
P14B1	†
P14B2	†
Waterleaf	
P24C10	1.0
P24B1	2.3
Mountain Pasque	
P158C10	3.5*
P158B3	†
P158B2	9.0

* Partial inhibition beyond recorded zone of complete inhibition.

† Inhibition in cup area only.

RESULTS

In vitro activity of plant extracts. Table 2 summarizes the results obtained using extracts of five plants against *Staphylococcus aureus* by the Oxford cup method. Saline extracts of the five plants were found to inhibit *Staphylococcus*. The extracts P14B1 and P14B2 of juniper and P158B1 of mountain pasque contained no diffusible antibacterial properties when tested in this manner. Butter- P158B2 extract of mountain pasque were found to inhibit completely the growth of all organisms in the plate. In later experiments this action was found to be due to a vapor. Waterleaf extract P24B1 was found to contain substances which were bacteriostatic. Later the same substance was found to have good anesthetic qualities *in vivo* (see Toxicity). P13B31 extract of sagebrush was found to have similar characteristics but not as pronounced.

Complete inhibition of all growth of the *Staphylococcus* in the seeded agar plates by extracts P16C10 and P16B69 of buttercup and P158B2 of mountain pasque—the Oxford cup method being used—indicated that there was good diffusion of the active principle, or that the inhibitory activity was due to the presence of vapor which was soluble. To ascertain the origin of this specific activity of the extracts, a small amount of the extracts was placed in the seeded petri plate without being in direct contact with the agar surface. These extracts were placed in the bottom of the inverted petri plate cover and after incubation at 37 C for 24 hours were observed to have no visible growth. To test the vapors' activity at greater distances than $\frac{3}{4}$ inch, seeded *Staphylococcus* plates were placed over the open tops of glass cylinders in which 6 to 10 drops of the extracts had been placed in the bottom. After incubation the plates were observed to contain no growth of the test organism in the exposed areas. Distances of from $\frac{3}{4}$ to 20 inches were used. As a control against capillary action of the liquids used, a seeded petri plate was suspenced in a bell jar 12 inches in diameter. A small amount of the buttercup extract P16C10 was placed in the bottom. The bell jar was incubated at 37 C for 18 hours. No growth occurred, indicating that the inhibitory action was due to the vapor. By extraction of the steam distillate of both plants, buttercup and mountain pasque, with ethyl ether and evaporation an oil was obtained. The oil exhibited the same properties as the saline extract when tested by the vapor method. The oil when placed in direct contact with the seeded agar inhibited the growth of the test organism, *Staphylococcus aureus*, in 5.0-cm diffusion areas.

The saline extract P16C10 and the vapor of buttercup were tested against *Penicillium* and *Aspergillus* species of molds, *Micromonospora* (Waksman), 5 species of *Streptomyces*, *Eberthella typhosa*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus viridans*. The methods described for the screening tests were used. The growth of all organisms was completely inhibited by the action of the vapor in the exposed areas. The activity of the extract produced inhibition-diffusion zones ranging from 3 to 5 cm on gram-positive organisms and 9.0 cm or complete inhibition on gram-negative organisms and molds.

The bacteriostatic and bactericidal activity of the P16C10 extract of buttercup is summarized in table 3. From the results of the experiments, the inhibitory effect of the saline extract appears to be bactericidal for gram-negative organisms and bacteriostatic for gram-positive organisms. Dilutions higher than 1:5,400 of the crude saline extract failed to inhibit the growth of gram-negative organisms. Concentration or purification of the active principle undoubtedly would greatly increase the dilution factor.

TABLE 3

Bactericidal and bacteriostatic effect of buttercup extract P16C10 on gram-negative and gram-positive bacteria

TUBE NO.	EXTRACT DILUTION	BACTERIA													EXTRACT CONTROL	
		S. aureus		*E. typhosa*		*C. diphtheriae*		*P. aeruginosa*		*S. viridans*		*D. pneumoniae*		*C. sporogenes*[3]		
		1	2	1	2	1	2	1	2	1	2	1	2	1	1	2
1	1:1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	1:3	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
3	1:5	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–
4	1:9	–	++	–	–	–	–	–	–	–	+	–	+	–	–	–
5	1:160	+	+++	–	–	–	–	–	–	–	++	–	++	–	–	–
6	1:320	+	+++	–	–	+	++	–	–	+	++	+	++	++	–	–
7	1:680	+	+++	–	–	+	+++	–	–	+	+++	+	+++	++	–	–
8	1:1,360	+	+++	–	–	+	+++	–	–						–	–
9	1:2,720	+	+++	–	–	+	+++	–	–						–	–
10	1:5,440	++	+++	–	+	+	+++	+	–						–	–
11	1:10,000	++	+++	++	+++	++	+++	++	+++						–	–
12	1:20,000	++++	++++	++++	++++	++++	++++	++++	++++						–	–
13	Organism Control	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	–	–

–, no growth; +, slight; ++, fair; +++, moderate; ++++, profuse.

1. Visual and microscopic appearance.
2. Growth on blood agar—4 mm loop.
3. Agar tubes.

The inhibitory action of the plant extracts against *P. gallinaceum* was tested by mixing 1:1 the extract and parasitized blood, previously diluted so that each ml contained 8 × 10^6 parasites. These mixtures were incubated at room temperature for 6 hours. One-quarter ml (10^6 parasitized cells) of the mixture was given intravenously to each chick. Three 2-week-old chicks were used in each test. The results of the experiment are presented in table 4. All the substances tested in this manner were lethal to the parasite. The control animals succumbed to malaria infections after receiving parasites treated in a similar manner with saline. The pH of each extract was adjusted to approximately 7.

Fresh-water protozoa (*Paramecium multinucleatum*, *Tetrahymena gellei*, and *Euglena* sp.) in clone cultures were found to lose their motility when placed in varying amounts of the saline extract P16C10 (buttercup). *Euglena* was found to lose its motility in less than 10 minutes in all tubes. *Paramecium* lost activity in the first tube within 10 to 15 minutes, with complete loss of motility in all tubes in 30 minutes. *Tetrahymena* appeared motile at the end of 20 minutes in the first tube, with no activity observed after 30 minutes. This protozoan remained active and motile in the ninth tube containing 0.2 ml of the extract at the end of 40 minutes, but no motility was observed in that tube or the tenth tube at the end of 60 minutes. The first tube contained 50 per cent solution of the extract and so on to the tenth tube which contained 9 per cent of the extract.

TABLE 4

In vitro effect of saline extracts of plants on Plasmodium gallinaceum

PLANT EXTRACT	NO. CHICKS	HOURS INCUB.	DIED	BLOOD POS.	DAYS OBSERV.	CONTROLS		EXO. FORMS	
						Blood pos.	Died	Test	Controls
Buttercup									
P16C10	3	6	0	0	40	3	3	0	0
P16C10b*	3	6	0	0	40	3	3	0	
Mountain pasque									
P158C10	3	6	1	0	33	3	3	0	1
Waterleaf									
P24C10	3	6	0	0	30	3	3	0	0
Sagebrush									
P13C10	3	6	1	0	30	3	3	0	0

* Infusion.

Toxicity tests. The amounts of crude saline and refined extracts that chickens and mice are able to tolerate are summarized in table 5. Observations were made at 10 and 60 minutes and at 24 hours. No attempts were made to determine the cumulative effects of the extracts as this could be observed in the *in vivo* experiments. The acute toxic symptoms helped to determine the levels which could be used in the *in vivo* tests.

The extracts, saline concentrate P24C10a and P24B1 of the waterleaf plant, and P13B31 extract of sagebrush were found to anesthetize animals receiving them. Waterleaf extract P24B1 produced (0.5 ml, i.p.) complete anesthesia for 4 to 5 hours when injected into mice. This same extract when given intravenously (0.5 ml) to chicks produced anesthesia and appeared to produce no toxic symptoms. P13B31 extract of sagebrush in a 1 per cent solution was toxic for mice if given intraperitoneally in 1-ml amounts. The animal went into a deep coma within a minute after injection and died within 7 minutes. The mice re-

TABLE 5

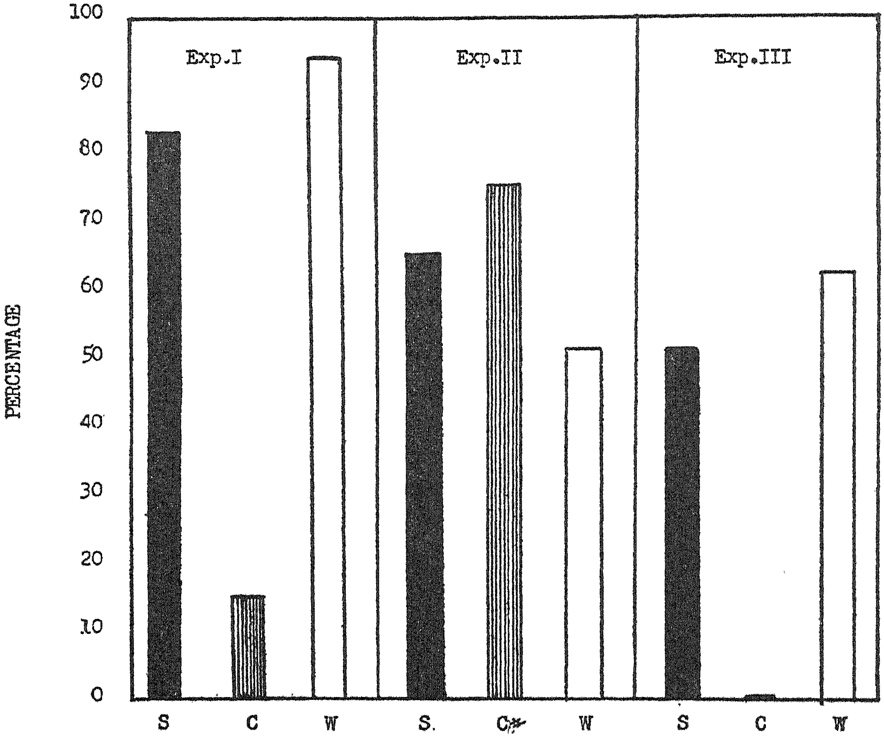
Toxicity of extracts of plants in chickens and mice

PLANT EXTRACTS	CHICKENS						MICE				REMARKS
	Intraperitoneal		Subcutaneous		Intravenous		Intraperitoneal			Subcutaneous	
	0.5 ml	1.0 ml	0.5 ml	1.0 ml	0.5 ml	1.0 ml	0.25	0.5 ml	1.0 ml	0.5 ml	
Buttercup											
P16C10	A	A	A	A			A	A	A	A	
P16B69			A	A							
P16B90			A	A			A	A	A	A	
Sagebrush											
P13C10			A	A			A	A	A	A	
P13B31—1%	A	D	A	D	A	D	A	A	D	D	Coma 0.5 i.p.
P13D24—1%	D	D	A	D			A	A	D	A	
P13C10a			A				D	A	A	A	0.25 i.p. Trauma
P13B31 1:400			A	A			A	A	A	A	0.5–1.0 i.p. Stagger
Waterleaf											
P24C10a	D	D	A	D			A	D	D	A	Partial paralysis before death i.p.
P24B1					A	A	A	A	A	A	Coma 0.5–1.0 i.p.
Mountain pasque											
P158C10	A	D	A	A			A	D	D	A	Subcutaneous blisters, chicks
P158B1a							D	D	D	A	
P158B1b							D	A	A	A	0.25 Trauma
P158B3			A	A			A	A	A	A	Subcutaneous blister
Juniper											
P14C10	A	D	A	A			D	A	A	A	0.25 Trauma
P14B1							A	A	A	A	
P14B2							A	A	A	A	
P14C10b			A				A	A	A	A	

A, alive after 24 hours.
D, died.

minutes. This stage lasted 5 to 10 minutes, at which time the animals began to revive. Further pharmacological and physiological studies are being undertaken on these two substances.

In vivo tests. A reduction in the total blood parasite counts of the treated groups as compared to the untreated control chickens was observed in the screen tests using the crude saline extracts. The original purpose of this study was to investigate substances separated from plants which were active against the blood stages. Therefore, if the infected chickens showed a definite and dramatic decrease in blood parasites and longer survival time during treatment, the plant extract was considered to have potential possibilities even though the chicken later died of exoerythrocytic forms of the disease.



GRAPH 1. PERCENTAGE OF CHICKENS DYING WITH EXOERYTHROCYTIC FORMS AFTER TREATMENT WITH SAGEBRUSH EXTRACT P13C10 AND WATERLEAF EXTRACT P24C10

S, sagebrush; C, controls; W, waterleaf. * —Quinine-treated controls.

Sagebrush extracts P13C10 and P13C10a and waterleaf extracts P24C10 and P24C10a were tried using larger groups of chickens. In this experiment a total of 87 chickens were used. In experiment I (graph 1) using saline extracts of the two plants, 10 out of 12 chickens treated with sagebrush and 11 out of 12 chickens of the waterleaf-treated group died, with exoerythrocytic forms observed in brain smears. Only 1 of the 6 chickens used in the control group was found to contain exoerythrocytic forms. These forms were observed as early as the tenth day of infection, with several birds of those specific test groups remaining completely free of the blood phase as determined by blood smears. The average day of death for chickens showing the presence of exoerythrocytic forms was 11.9 for those receiving sagebrush P13C10 and 11.7 with those receiving waterleaf P24C10. The one chicken in the control group succumbing to this form of the disease died on the sixteenth day.

Experiment I indicated that sagebrush P13C10 and waterleaf P24C10 extracts contained substances which were antimalarial in character in that less than 10 per cent of the waterleaf-treated and 20 per cent of the sagebrush-treated chickens died of the blood phase of malaria. The exoerythrocytic phase of the disease also appeared to be affected by the action of these two plant extracts. The presence of these forms was observed as early as the tenth day after inoculation, whereas the only chicken succumbing to the exoerythrocytic form in the control group died on the sixteenth day. With these findings in mind, a second experiment was done. To insure against the possibility of inoculating exoerythrocytic forms, donors with very early infections were used. The control group of chickens were to be treated with quinine HCl to ascertain if the plant extracts were enhancing the development of the exoerythrocytic forms of this infection. In experiment II, 9 of the 12 quinine-treated control group were observed with exoerythrocytic forms. All the birds succumbing to this form of the disease died between the seventeenth and nineteenth day of infection. Two chickens in the sagebrush group died on the eleventh day, and 5 on the sixteenth to the nineteenth day, with exoerythrocytic forms being observed in all brain smears. Six of the 12 chickens treated with waterleaf extract died, showing the presence of exoerythrocytic forms. The earliest death of this group with these forms did not occur until the nineteenth day.

Saline extract concentrates of the two plants, waterleaf P24C10a and sagebrush P13C10a, were used in experiment III (graph 1). With sagebrush, 4 out of 9 chickens died with exoerythrocytic forms, and those treated with waterleaf showed these forms in 5 of the 9 chickens. No exoerythrocytic forms were observed in the control group.

The survival time of the chickens treated with sagebrush extract in this group was found to be between 18 to 24 days, with one chicken remaining alive after becoming blood-parasite-negative; with waterleaf extract it was found to be between 13 to 24 days, the majority dying after the eighteenth day of infection; whereas in the control group all chickens had succumbed to blood infections between 8 to 11 days.

Further evidence of the antimalarial effect of sagebrush P13C10 and waterleaf P24C10 was shown by the fact that the parasite counts remained negative or less than 1 per cent throughout the infection, with death being due to exoerythrocytic forms. The blood smears were positive in the control chickens 4 to 5 days before they were positive in the test groups. The antimalarial effect was also observed in chickens with 50 to 70 per cent parasitemia. In these, continued treatment reduced the parasite count.

Extract P13B24 of sagebrush when tested as an antimalarial agent appeared to be active against the blood phase by reducing the parasite count in the screen test group, whereas extract P13B31 had no effect on the course of the infection in chickens. The latter extract, when given intraperitoneally, did cause the chickens to become stuporous. The antimalarial activity of P13B24 was further tested on a larger series of infected chickens. Three groups of 16 each were used. The first group received the drug 2 days before infection, the second group was treated as the chickens became positive, and the third group was the untreated group used as a control. In the pretreated group (I), 2 chickens died before becoming blood-positive and 4 died showing the presence of exoerythrocytic forms. The parasite counts of 5 chickens in this group remained less than 1 per cent or were reduced, 4 of these dying with exoerythrocytic forms. In group II, treated as they became positive, the results were very similar to those found in the chickens of the control group with the exception that 1 chicken died on the fourteenth day showing the presence of exoerythrocytic forms. All chickens in the control group had succumbed to the blood phase of the disease by the thirteenth day of infection.

Saline extract P14C10 of juniper, P158C10 of mountain pasque, and P16C10 of buttercup used as therapeutic agents against *P. gallinaceum* were found to contain no antimalarial activity when tested on larger groups of chickens. Refined extracts P158B1 and P158B3 of mountain pasque and P16B69 and P16B90 of buttercup were also found to have no apparent effect on chicken malaria.

Bacterial. The antibacterial effect *in vivo* of the saline and refined extracts was tested against *Diplococcus pneumoniae.* White Swiss mice were inoculated intraperitoneally with a 6-hour broth culture of type 19 pneumococcus. One hour after inoculation mice were injected with 0.5 ml of the extracts intraperitoneally and twice daily thereafter. Those animals alive after 24 hours were given further treatment. Five mice were used in the screening tests.

Of all the extracts tried (table 1) only one showed any action against the pneumococcus infection. The P158B3 extract of mountain pasque protected all mice at the end of 24 hours. All control animals were dead in the same period of time. Two mice of this screening group died in 48 hours with the other 3 remaining alive. Type 19 pneumococcus was isolated from the 2 mice dying in the treated group and from the control animals. To ascertain the *in vivo* activity of this drug in a larger group of animals, 50 mice were infected with type 19 pneumococcus from a 12-hour culture. The mice were divided into two groups of 25 mice each. One group was treated 1 hour after inoculation and twice daily thereafter. The second group was used for control and received no treatment. Six hours after inoculation all mice in the control group had succumbed to the pneumococcus infection. In the treated group all mice were alive at 6 hours, with 5 deaths occurring at the end of 8 hours. The remainder of the animals remained alive.

DISCUSSION

The foregoing experiments show that plants have distinct inhibitory substances

(*Ranunculus occidentalis*) and mountain pasque ([illegible]), tested for inhibitory substances by the petri plate method appeared to have very good diffusion. In some tests it was observed to have diffused throughout the seeded agar causing complete inhibition of growth of all organisms inoculated into the medium. Seegal and Holden (1945) have reported similar findings with buttercup, demonstrating definite inhibitory activity on a large series of microorganisms. As these plants, buttercup and mountain pasque, appeared far superior to any others tested, it was felt that they should be examined for a vapor which might prove to be soluble and have inhibitory characteristics. Such vapors were found. Fresh plants were always used in preparing all extracts. Buttercup and mountain pasque are closely related species in that they are of the same family, *Ranunculaceae*.

The P13C10 and P13B24 extracts of sagebrush and the P24C10 extract of waterleaf protected 50 per cent or more chickens during the blood phase of *P. gallinaceum* malaria. No apparent effect was observed on the exoerythrocytic forms. It was noted that the crude saline extracts (P13C10 and P24C10) protected larger numbers of infected chickens than the refined extracts of the plants.

The anesthetic activity of P13B31 extract of sagebrush and the P24B1 and P24C10a extracts of waterleaf was encountered when these extracts were being tested for toxicity. A more detailed report will follow at a later date on the pharmacological and toxicological aspects of these drugs.

It was felt by the authors that the plants which were observed to show activity against the blood phase of the malaria parasite would most likely be active against bacterial infections. Our results did not bear out this hypothesis. The P158B3 extract of mountain pasque was the only plant extract which protected animals against bacterial infections.

No attempt has been made at this writing to ascertain the specific chemical activity of any of these extracts.

SUMMARY

The saline extracts of five plants (buttercup, sagebrush, dwarf waterleaf, mountain pasque, and juniper) out of more than 200 tested were found to have antibacterial and antimalarial activity by *in vitro* methods.

One plant, buttercup, was found to inhibit the growth of many gram-negative and gram-positive bacteria, fungi, soil organisms, and nonpathogenic protozoa by *in vitro* methods.

Mountain pasque and buttercup were found to contain vapors which were bacteriostatic and bactericidal. Volatile oils separated from the same plants were found to exhibit similar activities.

An extract of mountain pasque was found to protect mice heavily infected with the pneumococcus.

Two plants, sagebrush and dwarf waterleaf, contained substances that protected chickens during the blood phase of malaria.

An incidental observation revealed that the same two plants possessed anesthetic properties in chickens and mice.

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THE POTENTIATION OF TETANUS TOXIN BY BROTH AND SERUM[1]

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Years ago Ricketts and Kirk (1906), Marie (1914), Bronfenbrenner (1924), Coleman (1924), Wagner, Meyer, and Dozier (1925), Jensen (1926), and Zuger, Hollander, and Friedemann (1939) reported that botulinus and tetanus toxins are potentiated by a number of substances. Among these, nutrient broth and blood serum are of particular interest, the former because it is a constituent of all impurified tetanus toxins, the latter because some problems require the quantitative determination of tetanus toxin in blood. In the course of experiments with tetanus toxin it occurred to us that neglect of this potentiation effect might cause serious errors in the evaluation of the potency of tetanus toxin. It was felt, therefore, that concerning this phenomenon more information was needed than is available from the scanty data in the literature.

In the first place, this added information pertains to the quantitative aspects of the potentiation effect. The majority of the above-mentioned papers contain no data concerning the extent to which tetanus toxin is potentiated by undiluted broth or serum. What is more important, it is unknown how far these substances can be diluted and still give a marked potentiating effect. On the basis of the available information it is impossible, therefore, to predict whether the titration of tetanus toxins will be affected by the broth content of the limiting dilutions.

Experiments with a large number of tetanus toxins led to the unexpected observation that some toxins are very strongly potentiated by broth and serum but that other toxins are not potentiated at all. It was further observed that the potentiation phenomenon is very marked in some animal species but that it is absent in others.

A great deal of work was devoted to attempts at elucidating the mechanism of the potentiation phenomenon. Although, thus far, the results of these experiments were unsatisfactory, we are at least in position to decide the question whether broth and serum actually potentiate the toxin or whether, as has been claimed by some authors, they serve merely as buffers which prevent the deterioration of the toxin in saline. On the basis of our experimental data the titration of tetanus toxin under various conditions will be discussed.

METHODS AND MATERIAL

Toxins 1175 H, 641 B, 1346, and 1375 were placed at our disposal through the courtesy of the Lederle Laboratories, Inc. We are indebted for toxin 103 to the Laboratories of the New York City Health Department. Toxins G, 8, 3, 2, 22, H, 4, 7, 5, 15, 6, A, B, C, J, K, D, E, F, and L were prepared in our laboratory.

With few exceptions, all dilutions of toxin were made either in broth, serum, or saline. A separate pipette was used for each dilution. In experiments with toxin 1175 H we observed a very marked pipette error. In experiments with other toxins, however, it did not make any difference whether or not the pipettes were changed.

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EXPERIMENTAL

Quantitative aspects of the potentiation phenomenon. Experiments with tetanus toxin 1556 in mice may serve as an illustration of the potentiating effect. Guinea pig serum, broth, and some constituents of broth were examined. As may be seen from table 1, guinea pig serum has the strongest potentiating effect (64 times); then follows broth (32 times). Difco peptone potentiates as strongly as broth, whereas Witte peptone has a weaker effect, but the potentiating effect is not exclusively due to the peptone content. Savita broth without peptone also potentiates markedly. Whether this effect is due to a single substance or to a variety of substances is still undecided.

TABLE 1

Potentiation of tetanus toxin 1556 by guinea pig serum, nutrient broth, broth without peptone, Difco peptone, and Witte peptone

One-tenth ml of the toxin dilutions was injected into the thigh muscles of white mice weighing 20 g

DILUTIONS OF TOXIN 1556	SALINE	GUINEA PIG SERUM	SAVITA BROTH WITH 1% DIFCO PEPTONE	SAVITA BROTH WITHOUT PEPTONE	DIFCO PEPTONE 1%	WITTE PEPTONE 1%
1:2,000	3	3	—	3	1	2
1:4,000	0	3	2	3	2	2
1:8,000	0	3	2	3	2	2
1:16,000	0	3	3	3	2	3
1:32,000	—	3	4	3	5	L.T. 2
1:64,000	—	3	4	L.T. 1	5	L.T. 2
1:128,000	—	3	L.T. 2	L.T. 3	L.T. 1	L.T. 2
1:256,000	—	G.T. 4	L.T. 2	L.T. 3	L.T. 2	L.T. 2
1:512,000	—	—	0	—	—	—

Numerals indicate day of death.
G.T. = General tetanus, numerals indicating day of onset.
L.T. = Local tetanus, numerals indicating day of onset.
0 = No symptoms.
— = Not done.

The following experiments were likewise conducted with toxin 1556, but the broth was examined undiluted and in the dilutions 1:10, 1:100, and 1:1,000. As may be seen from table 2, the potentiating effect of a broth dilution of 1:10 is just as strong as the effect of undiluted broth. A dilution of 1:100 diminishes the effect by only one-half. But even in a dilution of 1:1,000 broth still has a marked potentiating effect. The significance of these results for the titration of tetanus toxins will be discussed below.

Differences in the potentiability of tetanus toxins. Differences in the potentiability of tetanus toxins were uncovered by the following experiment: We tried to examine the absorption of tetanus toxin from the intraventricular fluid into the general circulation. Twenty lethal doses of toxins 1175 H and 103 were injected intracerebrally in guinea pigs weighing 250 g, and after various intervals samples of blood were taken and titrated for the presence of tetanus toxins in white mice weighing 20 g. The total amount of toxin 103 recovered from the blood was slightly less than the amount injected. In the experiment with toxin 1175 H,

TABLE 2

Potentiation of tetanus toxin by various dilutions of broth

One-tenth ml of the toxin dilutions was injected into the thigh muscles of white mice weighing 20 g

DILUTIONS OF TOXIN 1556	SALINE	BROTH UNDILUTED	BROTH 1:10	BROTH 1:100	BROTH 1:1,000
1:500	2	—	—	—	—
1:1,000	7	—	—	—	—
1:2,000	L.T. 3	—	—	—	6
1:4,000	0	—	—	1	6
1:8,000	—	—	1	2	L.T. 1
1:16,000	—	5	2	4	L.T. 1
1:32,000	—	5	4	L.T. 1	—
1:64,000	—	L.T. 3	L.T. 1	L.T. 1	—
1:128,000	—	L.T. 3	L.T. 1	L.T. 1	—

Numerals indicate day of death.
G.T. = General tetanus, numerals indicating day of onset.
L.T. = Local tetanus, numerals indicating day of onset.
0 = No symptoms.
— = Not done.

TABLE 3

Difference in potentiability of various tetanus toxins by guinea pig serum

One-tenth ml of the toxin dilutions was injected intramuscularly in white mice weighing 20 g

TOXIN DILUTIONS	TOXIN 103		TOXIN DILUTIONS	TOXIN 1175 H	
	Saline	Serum		Saline	Serum
1:500	5	—	1:1,600	4	2
1:1,000	6	5	1:3,200	L.T. 2	3
1:2,000	8	5	1:6,400	0	4
1:4,000	0	0	1:12,800	0	4
1:8,000	0	0	1:25,600	0	9
1:16,000	0	0	1:51,200	0	L.T. 2
1:32,000	0	0	1:102,400	—	L.T. 2

Numerals indicate day of death.
L.T. = Local tetanus, numerals indicating day of onset.
0 = No symptoms.
— = Not done.

been injected. Obviously this result could only be due to the potentiation of the toxin by the serum of the animal. We, therefore, carried out potentiation experiments with the two toxins.

TABLE 4

Potentiation of 31 tetanus toxins by guinea pig serum or broth

TOXIN	0.1 ML	TOXIN	0.1 ML	TOXIN	0.1 ML
G	1:50 1:50	641B	1:800 1:12,800	1346	1:1,000 1:4,000
8	1:100 1:200	5	1:800 1:6,400	T	1:1,600 1:25,600
3	1:100 1:400	15	1:800 1:1,600	K	1:1,600 1:12,800
2	1:200 1:1,600	3	1:800 1:3,200	D	1:1,600 1:12,800
1375	1:400 1:800	6	1:800 1:3,200	E	Z:1,600 1:12,800
22	1:400 1:400	A	1:800 1:6,400	F	1:1,600 1:12,800
H	1:400 1:6,400	B	1:800 1:25,600	641B	1:1,600 1:12,800
4	1:400 1:25,600	C	1:800 1:12,800	L	1:1,600 1:6,400
7	1:400 1:25,600	103	1:800 1:800	103	1:2,000 1:2,000
1346	1:500 1:16,000	103	1:1,000 1:1,000	1346	1:2,000 1:4,000
				1175H	1:4,000 1:128,000

One-tenth ml of each dilution of toxin was injected intramuscularly in white mice weighing 20 g. For each toxin the first row gives the lethal dose in saline. The second row gives the lethal dose in broth or serum. The lethal doses of toxins 3, 103, and 1346 were determined at various intervals after their preparation.

As may be seen from table 3, toxin 1175 H is strongly potentiated but toxin 103 is not potentiated at all. In the course of 2 years we repeated this experiment several times. Invariably toxin 103 was not potentiated either by broth or by serum.

In table 4 are recorded the results of potentiation experiments with a considerable number of tetanus toxins. It will be seen that there were 3 toxins (103, G, and 22) which were not potentiated at all and others, like toxins 8, 1375, 15, and 1346, which were only very slightly potentiated. Most of the toxins, however, were strongly potentiated.

The question presented itself whether these results are indicative of qualitative differences between the individual toxins or whether they are simply related to the strength of the toxins. It must be kept in mind that the lethal doses of weak toxins contain a considerable amount of broth, whereas very little broth is present in the lethal doses of strong toxins. Weak toxins, therefore, may fail to be potentiated because they are already more or less potentiated by the broth contents of their lethal doses. The results recorded in table 3 lend no support to this explanation. Toxin 103 was in the beginning one of the strongest toxins at our disposal. Moreover, toxins 1375, 22, H, 4, and 7 all had lethal doses of 0.1 ml of a 1:400 dilution. The potentiation effect, however, varied from 1:1 for toxin 22 to 64:1 for toxin 7. The differences in potentiability, therefore, indicate qualitative differences between the individual toxins. It may be remembered that other qualitative differences between tetanus toxins have been described in previous communications. Tetanus toxins were found to differ in the ratios of the lethal doses for the rabbit and the guinea pig (Friedemann, Zuger, and Hollander, 1939; Smith, 1943; Friedemann and Hollander, 1943) and in their avidities for nerve tissue (Friedemann and Hollander, 1943; Friedemann, Hollander, and Traub, 1946). It is unknown at the present time whether these various manifestations of qualitative differences are interrelated or independent of each other.

TABLE 5

Potentiation of toxin 1556 by broth in rabbits weighing 2,500 g

TOXIN 1556	DILUTIONS OF TOXIN MADE IN SALINE	DILUTIONS OF TOXIN MADE IN BROTH
0.1 ml undiluted	1, 4	4
" " 1:2.5	8, 9	4
" " 1:5	10, 16	2
" " 1:10	8, L.T. 3	5
" " 1:20	L.T. 3, —	G.T. 5
" " 1:40	—, —	0

Numerals indicate day of death.
G.T. = General tetanus, numerals indicating day of onset.
L.T. = Local tetanus, numerals indicating day of onset.
0 = No symptoms.
— = Not done.

The potentiation effect in different animal species. The experiments reported in the preceding sections were conducted on mice. The results are approximately the same in guinea pigs. It will be seen from table 5 that the potentiation of toxin 1556, which was very strong in mice and guinea pigs, is negligible in rabbits. We further observed no appreciable potentiation in cats. It is questionable, however, whether this result indicates fundamental differences between animal species. Both the rabbit and the cat are relatively resistant to tetanus toxin. The question, therefore, arises whether or not the broth content of the lethal dose is sufficient to produce maximal potentiation. This question will be answered only when media are available which contain no potentiating substances or when it is possible to conduct these experiments with purified toxins.

Some investigations on the mechanism of the potentiation effect. It may be questioned whether the term "potentiation effect" properly describes the observations reported in the preceding sections. According to Bronfenbrenner (1924), broth and serum act as buffers that prevent the spontaneous deterioration of botulinus toxin in saline. Halter (1936) and Neter (1942) have advanced the same explanation for the effect of broth and peptones on tetanus toxin. According to these authors, therefore, titrations of botulinus and tetanus toxins in broth, peptone, or serum would give correct results, but titrations in saline would be at fault. If this explanation be accepted, it is obviously unjustifiable to use the term "potentiation" in connection with our observations.

TABLE 6

Influence on toxicity of the time interval between preparation of dilutions of toxin and their injection

TOXIN DILUTIONS	A	B
1:500	1	1
1:1,000	1	2
1:2,000	2	3
1:4,000	L.T. 4	4

In the experiment recorded in column *A* the dilutions of toxin 1556 were injected immediately after their preparation. In the experiments recorded in column *B* the dilutions were kept at room temperature for 40 minutes before injection. All dilutions were made in saline, and 0.1 ml of each dilution was injected intramuscularly in white mice weighing 20 g.

Numerals indicate day of death.

L.T. = Local tetanus, numerals indicating day of onset.

In the following experiments evidence will be presented to the effect that our observations can be explained only by a real potentiation. In the first place, the dilutions of tetanus toxin in saline were injected immediately after their preparation. It is highly improbable that in the few minutes, at most, elapsing between the preparation of the dilutions and their injection enough toxin would be destroyed to explain our results. Furthermore, if the toxin deteriorated rapidly in saline, it would be inexplicable that, in the rabbit, dilutions of toxin in saline and broth have the same potency. The experiment recorded in table 6 shows that even if the dilutions of toxin are kept at room temperature for 40 minutes no loss of toxicity is observed.

To exclude the very unlikely possibility that the toxin might be destroyed immediately upon its dilution in saline, toxin 1175 H was first diluted in saline trol, dilutions of the toxin were made in saline and in serum from the outset. As may be seen from table 7, the titer is almost exactly the same irrespective of whether the toxin is diluted in broth from the outset or whether it is first diluted in saline 1:10, 1:100, or 1:1,000 and then broth added. This experiment shows conclusively that no toxin is destroyed immediately upon dilution in saline.

The same conclusion follows from neutralization experiments with antitoxin. If $\frac{31}{32}$ of the toxin were destroyed in saline, there should be a considerable difference in the antitoxin requirements for toxin diluted in saline or broth. We have shown in a previous communication (Zuger, Hollander, and Friedemann, 1939), and have confirmed the result time and again, that neither broth nor serum has any influence on the neutralization of tetanus toxin by antitoxin. There is, therefore, abundant evidence that the effect of broth and serum on tetanus toxin is a real potentiation phenomenon.

TABLE 7

Potentiation of tetanus toxin by guinea pig serum after preliminary dilutions in saline

TOXIN DILUTIONS	A	B	C	D	E
1:2,000	2	—	1	1	2
1:4,000	3	—	2	2	2
1:8,000	L.T. 3	—	2	2	2
1:16,000	0	2	2	2	3
1:32,000	—	4	3	3	5
1:64,000	—	7	4	6	5
1:128,000	—	8	6	6	5
1:256,000	—	L.T. 2	L.T. 2	6	L.T. 2
1:512,000	—	L.T. 3	L.T. 3	0	L.T. 3
1:1,024,000	—	0	0	0	0

In column *A* all dilutions of toxin 1175 H were made in saline. In column *B* all dilutions were made in guinea pig serum. In columns *C*, *D*, and *E* preliminary dilutions of 1:10, 1:100, and 1:1,000, respectively, were made in saline, and all further dilutions were made in guinea pig serum.

One-tenth ml of each dilution was injected intramuscularly in white mice weighing 20 g.

Numerals indicate day of death.

L.T. = Local tetanus, numerals indicating day of onset.

0 = No symptoms.

— = Not done.

We have conducted a large number of experiments in an attempt to elucidate the mechanism of this potentiation phenomenon. Since botulinus and tetanus toxins are potentiated but diphtheria toxin is not (Zuger, Hollander, and Friedeman, 1939), it was felt that this phenomenon might have something to do with the neurotropic character of the toxins. We thought of the possibility that potentiating substances might promote the entry of the toxins into the nerve endings. Our experiments in that direction, however, were inconclusive. We further investigated the influence of potentiating substances on neurotropic viruses. The potency of intramuscularly injected rabies virus in mice, however, was not enhanced by any of the potentiating substances. At the present time we are not in position to offer a satisfactory explanation of the potentiation phenomenon.

SUMMARY AND DISCUSSION

In the preceding sections evidence of the potentiation of tetanus toxin by broth and serum has been presented. The most important results may be summarized as follows:

(1) Broth, even in a dilution of 1:1,000, has a marked potentiation effect on some tetanus toxins.

(2) Some tetanus toxins are strongly potentiated by broth and serum, but other toxins are not potentiated at all. The potentiation phenomenon, therefore, reveals qualitative differences between tetanus toxins.

(3) The potentiation phenomenon is marked in mice and guinea pigs, but absent in cats and rabbits.

(4) Broth and serum do not act as buffers which prevent a deterioration of the toxin in saline. The effect of broth and serum on tetanus toxin is a real potentiation phenomenon.

Since it is a widely accepted opinion that tetanus toxin deteriorates rapidly in saline, it has been recommended that broth or peptone be added to the dilutions of toxin in order to prevent the latter's destruction. Our experiments show that this procedure introduces a serious source of error. The addition of broth or peptone enhances very considerably the potency of some tetanus toxins, but it leaves the potency of others unaffected. This method, therefore, gives not only too high values for potentiable toxins but may lead to an entirely erroneous estimation of the relative potencies of toxins.

Our experiments further stress the necessity of distinguishing between toxins and toxic filtrates. The latter contain substances which have a very marked effect on the potency of the toxins. We have seen that broth even in a dilution of 1:1,000 still has a marked potentiating effect. A correct and reliable determination of the potency of toxins, therefore, will be possible only under one of the following conditions: (1) that toxins are produced in media which contain no potentiating substances; (2) that the titrations are made with purified toxins; or (3) that only highly potent toxins are used for which the effect of the broth content on the titer is negligible. Of these procedures only the last one is practicable. Actually, the National Institute of Health, although for other reasons, prescribes that, for the production of toxoids, only toxins containing at least 10,000 minimal lethal doses per ml should be used. In research work, however, weaker toxins will sometimes be used, and it will be necessary to have in mind the fallacies which the use of such toxins may involve.

Another pertinent subject is the titration of tetanus toxin in blood. Ramon and Descombey (1931) studied the absorption of tetanus toxin from the intraventricular fluid into the blood. Abel, Evans, and Hampil (1936) investigated in a similar way the absorption of the toxin from muscle. A number of investigators (Knorr, 1898; Marie, 1897; Blumenthal, 1898) injected tetanus toxin intravenously and followed up its persistence in the blood.

The experiment reported in table 3 (also see text) shows that the results are very markedly determined by the potentiability of the toxins used in these experiments. The apparent titers in the blood may be from 16 to 64 times too high in experiments with potentiable toxins.

We wish to thank Miss Dorett Langstadt for valuable technical assistance.

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ELECTRON MICROSCOPY OF BACTERIUM TULARENSE[1]

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A recent report on the morphology of *Bacterium tularense* by Hesselbrock and Foshay (1945) suggested that a study made with the aid of the electron microscope would be of great value in the interpretation of the multiple morphological features of this organism. The great resolving power of the electron microscope would permit precise observation of the cell wall and would aid in the examination of forms the size and shape of which are not discernible by ordinary microscopic techniques.

The extremely high mortality and complete fragmentation of *Bacterium tularense* produced by sonic vibration (Coriell *et al.*, 1946) and the high mortality incurred by it during the lyophilization process (Penfield and Snyder, 1946) suggested that this organism might possess some unusual morphological features responsible for its marked susceptibility to these procedures.

METHODS

For the present investigation, three varieties of liquid media, (1) peptone broth (Snyder *et al.*, 1946), (2) gelatin hydrolyzate (Tamura and Gibby, 1943), (3) soybean hydrolyzate (Foshay, 1944), and a solid medium, glucose cysteine blood agar, were used for the cultivation of the organism. The incubation period was varied to obtain cultures with a variety of morphological forms.

Since Hesselbrock and Foshay (1945) could not differentiate between 43 virulent and avirulent strains on the basis of morphology, only two strains were used in the present study. They are designated as "Schu" and "Memp," and were obtained from Dr. Foshay. Both were fully virulent for laboratory animals and represent "typical" strains.

Various procedures were followed in preparing cultures for electron microscopic study. From solid medium suitable suspensions were prepared by one of the following techniques: (1) A small amount of surface growth was removed and suspended in sterile physiological salt solution or in sterile distilled water. The suspension was usually incubated at 37 C for 5 hours. (2) Three to four ml of sterile physiological salt solution were pipetted onto the surface of a slant culture, which was then allowed to incubate for a few hours at 37 C. (3) A small amount of surface growth was removed with a sterile wire and passed three or four times through a wire loop containing a film of sterile distilled water until a fairly uniform suspension resulted.

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[1] From studies conducted at Camp Detrick, Frederick, Md., and at the Johnson Foundation, University of Pennsylvania, from May, 1945, to September, 1945.

[2] Ensign, H(S), USNR.

[3] Captain, MC, AUS.

Suspensions from liquid media were used directly or diluted with one to two volumes of sterile distilled water. A drop of the suspension prepared by the previously described techniques was placed upon the collodion electron microscope mount by means of a capillary pipette or wire loop and allowed to dry in air (Marton, 1940).

Description of Electron Micrographs Presented in Figures 1 and 2

1. Saline suspension prepared from 24-hour glucose cysteine blood agar culture (Schu strain) and reincubated at 37 C for 20 hours. Cluster of coccoid forms showing peripheral arrangement of areas of greater electronic density.

2. Same. *Top:* Form showing peripheral area of greater electronic density and protoplasmic streamer or false "flagellum." *Middle:* Form suggesting binary fission. *Bottom:* Small coccoid form with extremely delicate cell wall.

3. Same. Large coccoid form with irregular area of greater electronic density and extremely delicate cell wall.

4. Saline suspension prepared from 96-hour glucose cysteine blood agar culture (Schu strain) and reincubated at 37 C for 5 hours. *Top:* Bacillary form and adjacent coccoid form. *Middle:* Filamented coccoid form with small spherical body at tip of filament. *Bottom:* Typical small globule of most prevalent size, 0.45 to 0.5 μ in diameter.

5. Peptone broth culture (Schu strain) incubated at 37 C for 24 hours. Large and small coccoid and bacillary forms. Note so-called "involution" form at right. (The greater electronic density of the forms in figure 1, nos. 5 and 6, is attributed to crystallization of salt within the cells.)

6. Same. Note variation in size of coccoid forms. Smallest form at upper right is approximately 210 mμ in diameter.

7. Same. Filamented bacillary form.

8. Distilled H_2O suspension prepared from 24-hour glucose cysteine blood agar culture (Schu strain) and reincubated at 37 C for 5 hours. Coccoidal form with single peripheral area of greater electronic density.

9. Same. Disintegrating coccoid forms. Note minute granular concentrations and "punched out" appearance.

10. Same. *Top:* Cluster of irregular coccoid forms with extremely delicate cell walls. Note another of the so-called "involution" forms. *Bottom:* Two coccoid forms. Contrast more dense form with disintegrating form at right.

11. Soybean hydrolyzate culture (Memp strain) incubated at 37 C for 4 days. Long delicate filament with rather dense granule near upper tip. Note small drumstick form at left.

12. Same. Large dumbbell-shaped structure with more dense spherical concentrations at terminal ends. Note possible early budding of form at left and filamented form at upper left.

13. Same. *Top:* Note crescentic shape of form at upper right and peripheral arrangement of areas of greater electronic density in the form directly below. *Bottom:* Large oval forms.

14. Same. Oval form with adjacent partially disintegrated cell. Note finely

beaded filament near center of electron micrograph and drumstick form at lower left.

15. Same. *Top:* Long delicate filament. *Bottom:* Oval form with long branching filamentous projection. The dense area is probably the result of salt crystallization within the cell. Note two minute forms directly above. The smaller is approximately 110 mμ in diameter.

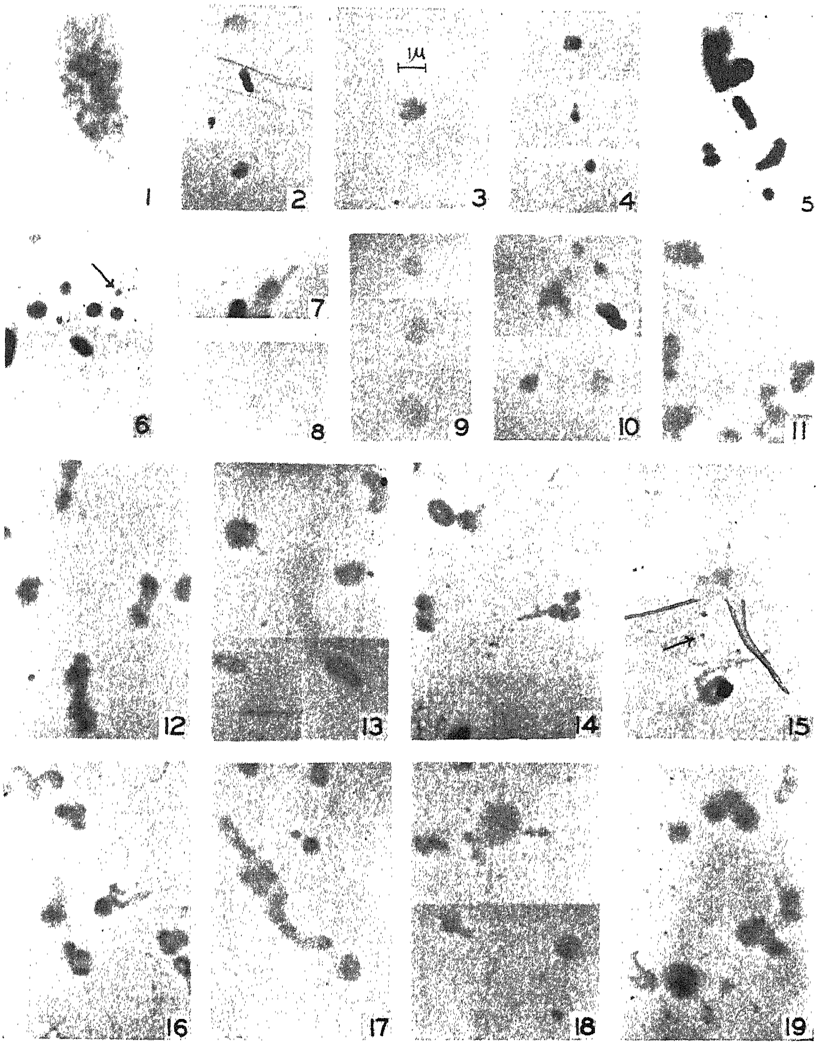

FIG. 1. ELECTRON MICROGRAPHS OF BACTERIUM TULARENSE
Reproduced with slight reduction from 6,800 diameters

16. Same. Large coccoid forms with peripheral areas of increased electronic density. Note fine filament arising from cell at lower left.

17. Same. Chain of large bacillary forms. Note bipolar arrangement of areas of greater electronic density within a number of cells.

18. Same. *Top:* Irregularly shaped coccoid mass with pseudopod projections. Structures of extremely low density such as this could be seen in most of our preparations. Suggests a ruptured cell with contents flowing into surrounding area. *Bottom:* Note crescentic arrangement of areas of greater electronic density within cell at right and irregular projection from cell at left.

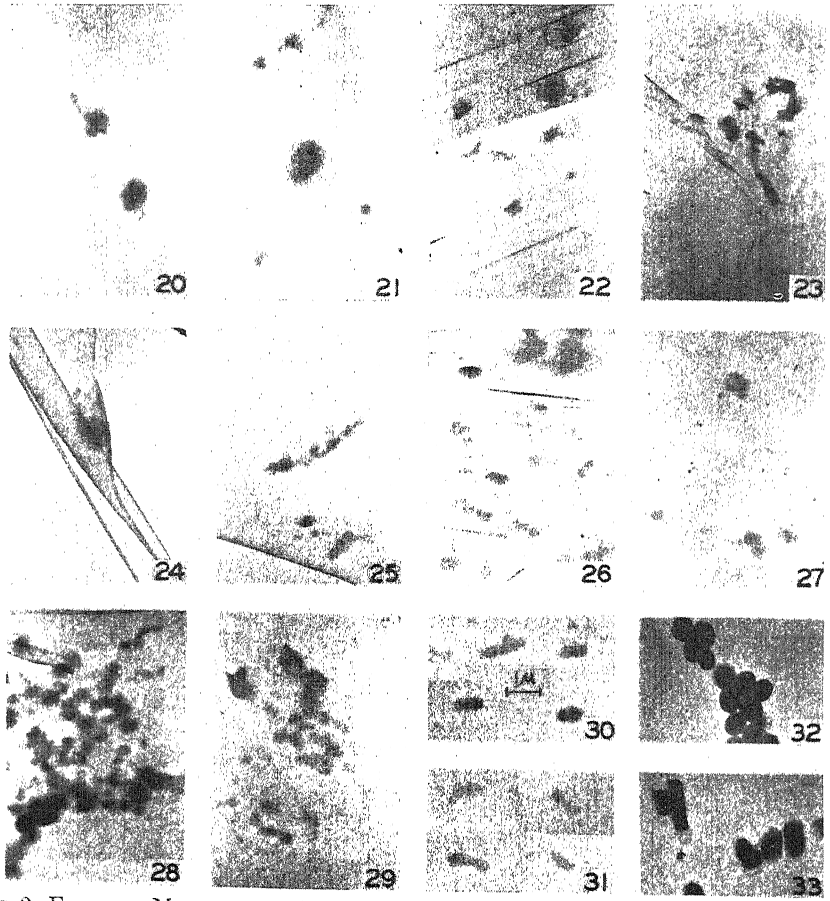

Fig. 2. Electron Micrographs: Nos. 20–29 Incl., Bacterium tularense (× 6,800); No 30, Washed Rickettsia rickettsiae (× 8,100); No. 31, Washed Murine Typhus Rickettsiae (× 6,800); No. 32, Staphylococcus aureus (× 6,800); No. 33, Eberthella typhosa "R" (× 6,800)

Reproduced with slight reduction from initial magnifications

19. Same. Comma-shaped form with large areas of greater electronic density at terminal ends.

20. Gelatin hydrolyzate culture (Schu strain) incubated at 37 C for 8 days.

Granular coccoid form with granular filamentous projection. Note apparent budding of form at right.

21. Same. Large coccoid form with extremely delicate cell wall. Note granules in the filament of the form above.

22. Distilled H_2O suspension prepared from colony (Schu strain) on glucose cysteine blood agar which had been incubated at 37 C for 3 days and allowed to remain at room temperature overnight. Folds in the collodion membrane are visible. *Top:* Large coccoid form showing patchy areas of greater electronic density. *Bottom:* Rupture of cell and dispersion of granular contents into surrounding area.

23. Same. Unusual shapes probably produced by traumatic distortion of cells.

24. Same. Large coccoid form. Contrast sharpness of collodion film edge with the nebulous character of the cell. Note that a part of the folded film edge can be seen through the semitransparent cell.

25. Same. Chain of coccoid forms. Note distribution of cellular debris produced by cellular rupture.

26. Same. Large coccoidal form in right center with protoplasmic streamer or false "flagellum." Note directly below evidence of sessile budding. Many disintegrating forms are visible.

27. Same. Another coccoid form suggesting budding. *Below:* Ruptured cell and protoplasmic streamer or false "flagellum."

28. Same. Cluster of coccoid, crescentic, and oval forms.

29. Same. Cluster of coccoid and oval cells of various sizes. Note the nebulous character of cells emphasizing the lack of density of this organism.

30. Washed *Rickettsia rickettsiae*, × 8,100. Note definite morphology and cell wall.

31. Washed murine typhus rickettsiae, × 6,800. Contrast the cell walls of the organisms shown in nos. 30 and 31 with the extremely delicate cell walls of *Bacterium tularense* shown in nos. 2, 3, 19, 20, and 21.

32. *Staphylococcus aureus*, × 6,800. Note density of these cells in contrast to low density possessed by cells of *Bacterium tularense* in nos. 1, 28, and 29.

33. *Eberthella typhosa* "R," × 6,800. Contrast cell wall and density of this organism with the cell wall and density of *Bacterium tularense*.

OBSERVATIONS

Most frequently observed during the study with both liquid and solid media was the small coccoid form 0.45 to 0.5 μ in diameter, although large coccoid, large and small bacillary, oval, minute, and filamented forms were often seen. Less frequently observed were bean-shaped, dumbbell, bizarre, and so-called "involution" forms which usually represented but a small portion of the total population. Finely filamented forms were more frequently observed in 5-day broth cultures than in younger broth cultures or cultures on solid medium. Occasionally delicate filaments attached to the cell, or broken and free, were observed. Frequently these filaments contained more dense minute concentrations described and termed "minimal reproductive units" by Hesselbrock and Foshay (1945). Areas of greater electronic density described by these authors as characteristic peripheral chromatin concentrations were often seen. The various morphological forms appeared singly, in diploform, or in short or long chain formation. No morphological differentiation could be made between the strains used in this investigation.

During the course of this study minute forms of 250 mμ or less in diameter were observed. Foshay and Hesselbrock (1945) reported that morphologic units of *Bacterium tularense* passed the 600 mμ Elford gradocol membrane but not the 500 mμ membrane and were therefore in the range of 300 to 350 mμ in diameter. These investigators postulated that units of smaller size exist. A minute morphological form, approximately 110 mμ in diameter, is shown in figure 1, no. 15. In figure 1, no. 6, a form approximately 210 mμ in diameter is shown. No morphological differentiation, with the exception of size, could be demonstrated between the minute forms and the usual coccoid forms.

Examination of the electron micrographs presented in figures 1 and 2 revealed that although electron micrographs were in sharp focus as evidenced by the edge of the collodion film seen in various preparations, the cells generally presented a hazy, nebulous appearance. *Bacterium tularense* seemed to possess an extremely delicate outer limiting structure (cell wall) of very low electronic density in contrast to the cell walls of various other organisms described by Wámoscher (1930) and reported to be "extremely solid, elastic, extensible, and enormously resistant to pressure." Usually the cell wall enclosed the cytoplasm so closely that the two were not distinguishable. The extremely delicate structure of the cell wall of *Bacterium tularense* is best illustrated in electron micrographs showing large coccoid and large bacillary forms the cytoplasm of which is unevenly distributed, allowing the cell wall to be more clearly visible. Usually it was difficult or impossible to differentiate the limiting edge of the delicate cell wall from the surrounding area. Protoplasmic streamers or false "flagella," indicating a break in the cell wall, were frequently observed. Although semitransparent cytolyzed cells were often observed and carefully examined, jagged lines of fracture which would indicate the presence of a solid or rigid cell wall were not demonstrated. These cytolyzed cells exhibited less opacity to the electron beam than did the mechanically cytolyzed *Bacillus subtilis* and *Bacillus anthracis* cells described by Mudd and associates (1941) and the mechanically cytolyzed streptococcal cells described by Mudd and Lackman (1941). Typical "ghost" cells showing only the cell wall were not observed.

From these observations it seems likely that the delicate structure of the cell wall of *Bacterium tularense* might at least partially account for the high mortality rates incurred during sonic vibration or lyophilization processes.

SUMMARY

The morphology of *Bacterium tularense* as determined with the electron microscope is in agreement with the systematic study of the morphology of this organism by means of vital staining techniques and dark-field examination reported by Hesselbrock and Foshay (1945).

In general *Bacterium tularense* possesses multiple morphological units including large and small coccoid and bacillary, oval, minute, filamented, bean-shaped, dumbbell, bizarre, and so-called "involution" forms. The suggestion of the existence of minute morphological units of less than 300 mμ in diameter was confirmed. The typical cell possesses little opacity to the electron beam and presents a semitransparent nebulous appearance. Critical examination for the presence of a cell wall revealed an extremely delicate structure of very low electronic density which possibly accounts for the low survival rate when subjected to sonic vibration or the lyophilization process.

ACKNOWLEDGMENT

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THE NUTRITION OF PHYTOPATHOGENIC BACTERIA

II. The Genus Agrobacterium

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The nutritional requirements of the tumor-inducing phytopathogenic bacteria and soil saprophytes which Conn (1942) has placed in the genus *Agrobacterium* have received somewhat more attention than have the requirements of most of the other groups of plant-disease bacteria. It is clear from the literature (Sagen, Riker, and Baldwin, 1934; Riker, Lyneis, and Locke, 1941; Hofer, 1941) that the crown-gall bacterium, *Agrobacterium tumefaciens*, and the common soil saprophyte, *Agrobacterium radiobacter*, grow well in solutions containing only ammonium or nitrate nitrogen and any of a number of carbon sources.

On the other hand, the remaining two species at present in this genus, the hairy-root organism, *Agrobacterium rhizogenes*, and the cane-gall bacterium, *Agrobacterium rubi*, are reported to have somewhat more complex nutritive requirements. For example, Sagen, Riker, and Baldwin (1934) summarize their study of *A. rhizogenes* by stating that it "seems to lack the ability of *P. tumefaciens* and *B. radiobacter* to utilize the simpler nitrogenous compounds." Similar observations are recorded for *A. rubi* by Pinckard (1935) and Hildebrand (1940).

Inasmuch as knowledge of the exact nutrition of this group may be useful in interpretations of the systematics and general physiology of the genus, as well as provide source material for eventual evaluation on the possible interrelationship of microbial nutrition and virulence (McNew, 1938; Van Lanen, Baldwin, and Riker, 1940), a study of the four *Agrobacterium* species was undertaken. At the same time some observations were made on the nutritive requirements of *Bacterium pseudotsugae* and *Agrobacterium gypsophilae*, two species which have been placed in an Appendix to *Agrobacterium* in the forthcoming sixth edition of *Bergey's Manual of Determinative Bacteriology*. In general, the objective was prompt, moderate growth in simple solutions of known composition, rather than development as rapid and luxuriant as possible in complex media.

I

Representative cultures were secured from investigators who had specialized in this group; specific information concerning sources is presented in the sections which follow. The purity of all cultures was checked by microscopic examination and by streaking serially two or more times on yeast extract agar and on glucose, yeast extract, and $CaCO_3$ agar from dilute aqueous suspensions of cells.

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[1] National Research Council Fellow, 1944–1946. On leave of absence from the Department of Biology, Brooklyn College, Brooklyn 10, New York.

The technical details were similar to those in the previous study in this series (Starr, 1946). All glassware was acid-washed. Media were prepared from reagents of the highest available purity. The basal medium[2] was the same NH_4Cl, glucose, and salts solution used before. At times, a commercially prepared HCl hydrolyzate of "vitamin-free" casein (SMACO brand) was used. In order to minimize the carry-over of nutrilites with the inoculum, test media were inoculated, by means of a capillary pipette, via a dilution flask. Cultures were incubated, often on a shaking machine to increase the rate of growth, at 25 to 28 C. Quantitative estimations of "turbidity" were made in the Evelyn photoelectric colorimeter using the 620 mμ filter, and the results are expressed in terms of "optical density" (2 − log galvanometer reading).

II

Agrobacterium radiobacter and Agrobacterium tumefaciens. The following 5 cultures of *A. radiobacter* were used in this study:

TR1 (Hofer's R1-1a), received from A. W. Hofer, Geneva, N. Y., in 1941.
TR4 (Hofer's 36), an old culture from F. Löhnis, identified by M. W. Beijerinck but not the latter's original culture. Received from A. W. Hofer, Geneva, N. Y., in 1943.
TR5 (Hofer's S-192), isolated by N. R. Smith from corn soil in 1927. Received from A. W. Hofer, Geneva, N. Y., in 1943.
PG.1.2 (Leonard's 1911S) and PG.1.3 (Leonard's 2012), received in 1945 from the collection of C. B. van Niel, Pacific Grove, Calif. Originally from L. T. Leonard, U. S. Department of Agriculture.

A. tumefaciens was represented by the following 14 isolates:

TT2 (Braun's B2) and TT3 (Braun's B6), received from A. C. Braun, Princeton, N. J., in 1941.
TT4 (Hofer's SCA-2), TT5 (Hofer's SCT-5 fff3), and TT6 (Hofer's SCA-1), received from A. W. Hofer, Geneva, N. Y., in 1943.
TT7 (Williams' A-1), TT8 (Williams' B-3), TT9 (Williams' H-100), TT10 (Williams' 5 Gly Fe), and TT11 (Williams' T3-1C-3), from the collection of I. M. Lewis; received in 1945 from O. B. Williams, Austin, Texas.
A-6, Cor, Wellesley, and W-1, received from D. G. R. Wyckoff, Wellesley, Mass., in 1946.

Most of these *A. tumefaciens* strains induced typical galls when inoculated[3] by pin pricks into the crowns of sugar-beet plants (Suit, 1933); however, TT5, TT10, and TT11 did not cause gall formation in repeated trials.

All cultures of *A. radiobacter* and *A. tumefaciens* grew luxuriantly within a day or two in the NH_4Cl, glucose, and salts basal medium, except that culture TT10 grew somewhat more slowly than the others. Larger crops, and slight increases in growth rate (marked in the case of TT10), resulted from the addition of 0.5 per cent of "vitamin-free" casein hydrolyzate to the basal medium. This stimulatory effect is due, in part at least, to the maintenance of a favorable hydrogen-ion concentration, close to neutrality, in the casein hydrolyzate medium. Cultures in the unsupplemented basal medium become acid rapidly, the pH dropping to the presumably growth-inhibiting level of about 4.2 in a day or two. The addition of 0.1 per cent of synthetic *dl*-glutamic acid to the basal medium also resulted in larger crops, although the increase was not so marked as with casein hydrolyzate, and caused TT10 to grow as rapidly as the other cultures.

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[2] Per 100 ml of basal medium: NH_4Cl, 0.1 g; KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; "trace" elements; distilled water; adjusted to pH 6.8 with NaOH. Separately sterilized, purified glucose (0.5 per cent) was added aseptically to the sterile salt solution.

[3] The co-operation of Dr. W. A. Campbell, Special Guayule Research Project, Salinas, California, in conducting the pathogenicity tests, is gratefully acknowledged.

A mixture of seven B vitamins[4] added to the casein hydrolyzate medium similarly increased, very slightly, the initial growth rate of all these cultures, probably, as McIntire, Riker, and Peterson (1941) have shown, because of the content of thiamine, riboflavin, and pantothenic acid. However, no further attention was directed to the exact evaluation of these but slightly stimulating nutrilites.

Agrobacterium rhizogenes. Four isolates of *A. rhizogenes* were studied:

TR7 (Hofer's C-1), received from A. W. Hofer, Geneva, N. Y., in July, 1943.
TR12, received from E. M. Hildebrand, Ithaca, N. Y., in July, 1943.
TR16 (Williams' T37), from the collection of I. M. Lewis; received from O. B. Williams, Austin, Texas, January, 1945.
C-10, received from A. W. Hofer, Geneva, N. Y., in January, 1945.

The virulence of these cultures was tested (Suit, 1933) by inoculating them into the crowns of sugar-beet plants by means of pin pricks. From 4 to 6 plants were used per culture in each trial, and the series was carried out three times. Culture TR7 was able to induce typical hairy-root symptoms most readily of the four isolates; TR12 and C-10 caused somewhat less severe hairy root in only part of the plants used, and the symptoms were rather delayed; TR16 was pathologically more like the crown-gall organism and always yielded galls rather than hairy root on sugar beet. This last culture was received as number T37 and may be descended from the culture of the same designation with which Hendrickson, Baldwin, and Riker (1934) obtained similar intermediate results.

Despite the varied pathogenicity, the four *A. rhizogenes* cultures behaved uniformly with respect to nutritive requirements. None of these cultures grew in the NH_4Cl, glucose, and salts basal medium, nor upon the addition of "vitamin-free" casein hydrolyzate. The addition of 0.5 per cent yeast extract to the casein hydrolyzate medium resulted in good growth. The yeast extract could be substituted by the mixture of seven B vitamins, and, by successively omitting one growth factor at a time from this mixture, biotin was inferred as the only active ingredient. In support of this inference, all four strains of *A. rhizogenes* grew when biotin was added to the casein hydrolyzate medium. In the presence of biotin the casein hydrolyzate could be replaced by 0.1 per cent of synthetic *dl*-glutamic acid. There was much slower growth in the basal medium supplemented only by biotin, and the final crops were decreased—probably, as indicated by pH measurements, because of acid production without a simultaneous neutralization by the alkaline decomposition products from the amino acids. Figure 1 shows the quantitative response to biotin in the glutamic acid medium. Biotin could be substituted by the hydrosulfate of 3,4-diamino-tetrahydro-2-thiophene-valeric acid, but not by pimelic acid.

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[4] *p*-Aminobenzoic acid, biotin, nicotinic acid, calcium pantothenate, pyridoxine HCl, riboflavin, and thiamine HCl. The vitamins and vitamin derivatives used in this study represent a generous gift from Merck & Co., Rahway, N. J.

The finding of an obligate biotin requirement for *A. rhizogenes* explains the difficulty encountered by Sagen, Riker, and Baldwin (1934), Riker, Lyneis, and Locke (1941), and Hofer (1941) in culturing this species in media which lacked peptone or yeast extract.

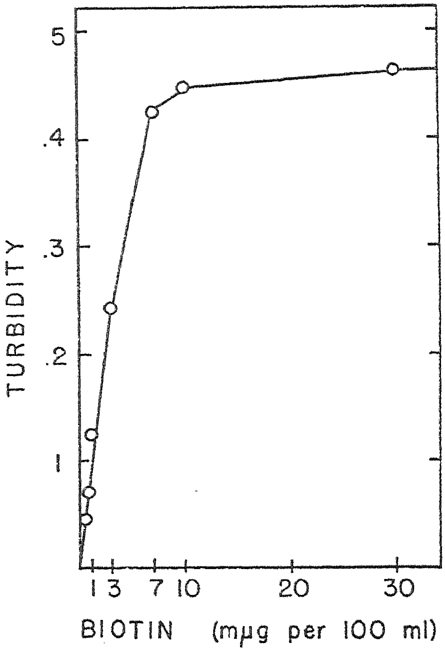

FIG. 1. RESPONSE OF AGROBACTERIUM RHIZOGENES TR12 TO BIOTIN IN THE GLUTAMIC ACID MEDIUM

The glutamic acid medium, supplemented by biotin in concentrations ranging from 0.1 to 100 millimicrograms (mμg) per 100 ml, was inoculated and incubated, with constant shaking, for 4 days at 28 C, at which time the "turbidity" was measured. The "optical density" at 100 mμg was the same as at 30 mμg.

The use of a calcium glycerophosphate medium for distinguishing *A. rhizogenes* from *A. tumefaciens* and *A. radiobacter* has been recommended. The hairy-root organism reportedly fails to grow (Riker, Banfield, Wright, Keitt, and Sagen, 1930; Sagen, Riker, and Baldwin, 1934), but the crown-gall bacterium and *A. radiobacter* make abundant growth. Inasmuch as it seemed likely that the failure of *A. rhizogenes* to grow in this biotin-free glycerophosphate medium merely mirrors its inability to develop in the absence of biotin and glutamic acid, the *A. rhizogenes* isolates were cultured from small inocula in the glycerophosphate medium[5] used by Riker *et al.* (1930), and with additions of biotin, of glutamic acid, and of both biotin and glutamic acid. These experiments may be summarized as follows: (1) There was no growth in the unsupplemented glycerophosphate medium or when glutamic acid alone was added. (2) There was slow growth when biotin alone was added. (3) There was good development in the glycerophosphate medium with both biotin and glutamic acid. The unsupplemented glycerophosphate medium supported good growth of all cultures tested of *A. tumefaciens* and *A. radiobacter*, entirely in accordance with the findings of previous investigators. There can be no doubt, then, that the *modus operandi* of the glycerophosphate medium test results from the fact that the nutrilite needs of *A. rhizogenes* are not satisfied by that medium.

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[5] Calcium glycerophosphate, 0.8 g; mannitol, 20.0 g; KNO_3, 5.0 g; NaCl, 3.8 g; KCl, 0.1 g; $MgCl_2 \cdot 6H_2O$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; distilled water, 1,000 ml.

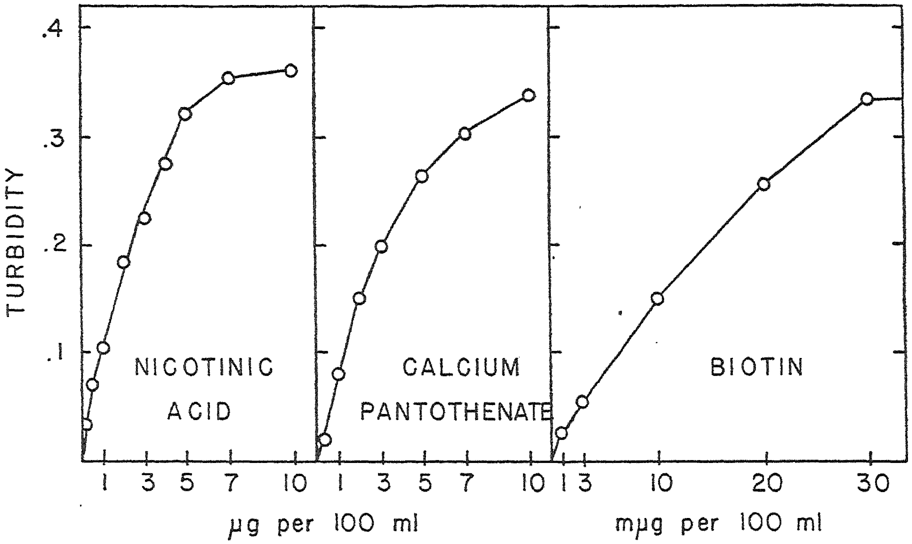

FIG. 2. RESPONSE OF AGROBACTERIUM RUBI TO NICOTINIC ACID, CALCIUM PANTOTHENATE, AND BIOTIN IN THE GLUTAMIC ACID MEDIUM

The response to nicotinic acid was measured in the presence of 10 μg per cent of calcium pantothenate and 100 millimicrograms (mμg) per cent of biotin; the response to calcium pantothenate, in the presence of 10 μg per cent of nicotinic acid and 100 mμg per cent of biotin; and the response to biotin, in the presence of 10 μg per cent each of nicotinic acid and calcium pantothenate. These media were incubated, with constant shaking, for 4 days at 28 C, at which time the "turbidity" was measured. The plotted values are the averaged "optical densities" of duplicate determinations.

This may be the place to comment on an experiment described by Hendrickson, Baldwin, and Riker (1934) in which the relation of the growth of *A. rhizogenes* to the oxidation-reduction potential of the medium was being considered. In one trial *A. rhizogenes* was cultured in an agar medium which was somewhat deficient in the necessary nutrilites, although sufficient growth factor was supplied by the agar so that some growth did occur. Hendrickson, Baldwin, and Riker (1934) go on to state that "when the oxidation-reduction potential of the medium was increased by the addition of 0.1 gram of potassium permanganate per liter . . . the hairy-root cultures either failed to grow or produced only slight growth below the surface." Inasmuch as biotin activity is destroyed by oxidizing agents (Melville, 1944), the inactivation of this needed factor might be considered as an alternative explanation of this experiment.

Agrobacterium rubi. Two isolates of this species were available for study; both (TR2, TR3) were obtained from E. M. Hildebrand, Ithaca, New York, in 1942, and both induced gall formation readily by inoculation, through pin pricks, into young stems of a bramble and into sugar-beet crowns. Neither of these cultures grew in the basal medium alone, or when "vitamin-free" casein hydrolyzate was added. By a process similar to that described above, it was learned that this species requires obligately three nutrilites for development in the casein hydrolyzate medium, viz., biotin, nicotinic acid, and calcium pantothenate. In the presence of these required growth factors, 0.1 per cent of synthetic *dl*-glutamic acid could replace the casein hydrolyzate. There was no growth in the basal medium plus the three vitamins alone. The response of *A. rubi* to each of the required vitamins in the glutamic acid medium is shown in figure 2. Biotin could be replaced by the hydrosulfate of 3,4-diamino-tetrahydro-2-thiophene-valeric acid, but not by pimelic acid; nicotinic acid was replaceable by nicotinamide; pantothenate was not replaceable by pantoyl 1-lactone, β-alanine, or a mixture of the two.

The obligate requirement of *A. rubi* for the three vitamins and glutamic acid throws some light on the poor growth obtained by Pinckard (1935), Hildebrand (1940), and Starr and Weiss (1943) in what can now be interpreted as inadequate media. It also explains the difficulty experienced by Hildebrand (1940) in attempting "fermentation" tests of this organism using the vitamin-free basal medium of the *Manual of Methods for Pure Culture Study of Bacteria* (Comm. Soc. Am. Bact., 1923–1936).

Agrobacterium gypsophilae and Bacterium pseudotsugae. As noted above, cultures of these species were included in this study because of their incorporation by H. J. Conn in an Appendix to the genus *Agrobacterium* in the sixth edition of *Bergey's Manual of Determinative Bacteriology*. The single culture of *A. gypsophilae* (TG1) was received from H. J. Conn, Geneva, New York, and was originally from N. A. Brown, Washington, D. C. Culture TG1 grew well in the unsupplemented NH_4Cl, glucose, and salts basal medium and was not particularly stimulated by the addition of casein hydrolyzate or yeast extract. These results are in accordance with the original description of this species, in which Brown (1934) reported good growth in several simple synthetic media.

Three cultures of *B. pseudotsugae* were used. These were isolated by the writer in 1945 from typical stem galls on Douglas fir material collected by H. N. Hansen, Berkeley, California, one of the describers of this species. The cultures isolated from this material were kindly identified by Dr. Hansen, but no pathogenicity tests were performed. These cultures did not grow in the unsupplemented basal medium, nor when casein hydrolyzate was added. By a process similar to that used for the foregoing species, it was determined that *B. pseudotsugae* requires biotin obligately for growth in the casein hydrolyzate medium. The casein hydrolyzate could not be replaced completely by glutamic acid alone, and no further study of amino acid requirements has been made.

III

Application of these findings to the taxonomy of this group would depend upon examining a larger sampling of these species to discover the range of variation with respect to exact nutritional requirements. In any case, the use of a nutrilite requirement as a determinative character in systematic microbiology must take into consideration the mutability of microorganisms in this regard. The potentialities in this group, however, appear promising in view of the consistent, reproducible results. Inasmuch as the literature recommends that the general fastidiousness of *A. rhizogenes* and *A. rubi* can aid in the identification of these species, it appears likely that the present disclosure concerning the exact nature of the specific nutrilite requirements would possess even more value in this connection.

The incorporation of the required nutrilites in basal media would make possible a study of the carbon metabolism of the fastidious species without possible interference from the yeast extract supplement used by Conner, Riker, and Peterson (1937) and Hofer (1941). Also, it would now be possible to determine, in adequately composed basal media, whether specific differences exist between the ability of the representatives of the genus *Agrobacterium* to utilize certain carbon compounds.

This knowledge might be used in the isolation of these species from plant materials, by preparing isolation media containing only the minimal nutrients for the species under investigation. In this way interference from contaminating organisms having more complex and different nutrient requirements would be avoided.

SUMMARY

The nutritive requirements of members of the genus *Agrobacterium* were determined under specified experimental conditions. By this means the previously reported ability of *A. radiobacter*, *A. tumefaciens*, and *A. gypsophilae* to grow in simple media was verified by using a medium containing NH_4Cl, other inorganic salts, and separately sterilized, purified glucose.

The inability of *A. rhizogenes*, *A. rubi*, and *Bacterium pseudotsugae* to grow in this medium was traced to obligate nutrilite requirements, viz., *A. rhizogenes*, biotin and glutamic acid; *A. rubi*, biotin, nicotinic acid, calcium pantothenate, and glutamic acid; *B. pseudotsugae*, biotin and some as yet unidentified component of "vitamin-free" casein hydrolyzate.

The utility of these findings in the systematics of the group is discussed.

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THE AMINO ACID COMPOSITION OF MICROORGANISMS

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Despite the extensive data that has accumulated on the chemical composition of microbial cells, information concerning microbial proteins, which are of major importance among cellular constituents, is fragmentary and, in part, inaccurate. This has been due primarily to inadequate and unreliable analytical methods for the determination of the basic protein units, the amino acids. The literature to 1926 on the amino acids in bacteria, yeasts, and fungi has been reviewed by Buchanan and Fulmer (1928). More recent papers which deal primarily with the amino acids of various species of *Azotobacter* are cited by Camien, Salle, and Dunn (1945). Similar information on marine and fresh-water algae is also available (Mazur and Clarke, 1938, 1942) as well as more recent data on yeasts (Sagara, 1930; Fink and Just, 1942; Block and Bolling, 1945).

In addition to recording some of the types and approximate quantities of amino acids in various microorganisms, early investigators attempted to determine whether the amino acid composition of a particular organism is constant or varies with environmental conditions, especially with the composition of the medium. Abderhalden and Rona (1905) found the same amino acids, namely, glycine, alanine, leucine, glutamic acid, and aspartic acid, in *Aspergillus niger* irrespective of whether the nitrogen source in a mineral, salt sugar medium was KNO_3, glycine, or glutamic acid. Also Tamura (1913*b*) found no significant difference in the amino acid composition of the proteins synthesized by *Mycobacterium lacticola* in nutrient broth, as compared to a protein-free medium consisting of mineral salts, ammonium lactate, asparagine, and glycerol.

Tamura (1914), on the basis of data obtained previously (1913*b*) which showed that *Mycobacterium tuberculosis* and *Mycobacterium lacticola* were rich in phenylalanine but free of sulfur-containing amino acids, suggested that it might be possible to classify microorganisms according to their chemical composition. Although an interesting idea, it rested on faulty data since cystine is present in *M. tuberculosis* (Johnson and Brown, 1922; Coghill, 1926). More recently, however, Mazur and Clarke (1938) reported that, among marine algae, the most primitive group, *Ulva*, lacked methionine, tyrosine, and lysine, and that those amino acids appeared in the foregoing order on ascending the evolutionary series. Cystine was absent from the fresh-water, blue-green alga, *Phormidium*, but was present in the marine algae. Arginine was absent from three out of five brown algae and from one of two green algae (Mazur and Clarke, 1942). It appears, therefore, that qualitative differences in amino acid composition may occur among microorganisms.

Recently, an accurate, specific, and sensitive microbiological method was developed for the determination of ten amino acids, namely, histidine, arginine, lysine, leucine, isoleucine, valine, methionine, threonine, tryptophane, and phenylalanine, in natural materials (Stokes, Gunness, Dwyer, and Caswell, 1945). The method permits simple and rapid analysis of large numbers of small-sized samples with an accuracy of generally ±10 per cent. Thus one gram of dry material is sufficient for the determination of all ten amino acids, and one experienced individual can analyze a half-dozen proteins in little more than a week. In the present investigation the method has been used to extend previous investigations on the amino acid composition of microorganisms; to determine with greater accuracy than was formerly possible, although in a necessarily limited manner, whether fundamental differences in amino acid content, such as described above for algae, exist among bacteria, yeasts, actinomycetes, and fungi; and finally to determine to what extent, if any, variations in medium and conditions of growth influence the amino acids of microorganisms.

After completion of the present investigation, data appeared in print on the amino acid content of various lactic acid bacteria and *Escherichia coli* as determined by microbiological methods (Camien, Salle, and Dunn, 1945).

CONSTANCY OF AMINO ACID COMPOSITION UNDER UNIFORM CONDITIONS OF GROWTH

In order to be able to assess the significance of possible differences in the amino acid composition of different microorganisms and of the same organism under varying cultural conditions, it was deemed necessary to determine first whether the amino acid composition of microbial cells is constant on repeated cultivation in the same type of medium and under the same conditions of growth. *Staphylococcus aureus*, *Aspergillus niger*, and *Streptomyces griseus* were each grown, three successive times, in a medium consisting of 0.5 per cent each of bacto beef extract and bacto peptone and 1 per cent glucose in distilled water. The glucose was essential for good growth of the fungus. The medium, unadjusted, had a pH of 6.8. The broth was distributed in 400-ml quantities into 2-L Erlenmeyer flasks. All inocula were grown in the same medium. The cultures were incubated at 30 C without agitation, except that *Staphylococcus aureus* was maintained at 37 C. The latter organism was harvested after 1 day, *Aspergillus niger* after 3 days, and *Streptomyces griseus* after 5 days, at which times it was estimated, from previous experience, that substantially maximum growth had occurred. The culture of *Aspergillus niger* and *Streptomyces griseus* consisted of mycelium and numerous spores. The cells were washed with water to remove any adhering medium, dried at 105 C overnight, weighed, and thoroughly ground in a mortar to give uniform preparations for subsequent amino acid analyses. Care was taken to keep all conditions as uniform as possible in preparing the successive batches of organisms. The amino acid determinations were conducted by the method described previously (Stokes *et al.*, 1945) on acid or alkaline hydrolyzates of aliquots of the dried microbial preparations. Their nitrogen contents were also determined by the Kjeldahl method using $CuSO_4$, K_2SO_4, and H_2SO_4 for digestion.

The results (table 1) demonstrate, unequivocally, that each of the three representative organisms when cultivated repeatedly under essentially identical conditions gave, generally within 10 per cent, the same dry weight yield, nitrogen content, and percentages of the ten amino acids. It is clear, therefore, that the amino acid composition of an organism is a stable and characteristic property of the cell under fixed conditions of growth.

AMINO ACID COMPOSITION OF REPRESENTATIVE MICROORGANISMS

It is evident from table 1 that there are great differences in the quantities of various amino acids present in the bacterium and actinomycete compared to those in the fungus and that differences exist, although to a lesser extent, between the quantities in the bacterium and actinomycete. Part of the data in table 1 is repeated in table 2 in conjunction with analyses of two additional bacteria, *Escherichia coli* and *Bacillus subtilis;* two yeasts, *Saccharomyces cerevisiae* and *Rhodotorula rubra;* and two additional fungi, *Rhizopus nigricans* and a penicillin-producing strain of *Penicillium notatum.* All these microorganisms were grown in the nutrient broth glucose medium and under the same conditions described above for *Staphylococcus aureus*, *Aspergillus niger*, and *Streptomyces griseus.* The bacteria were harvested after incubation for 1 day at 37 C, the yeasts after 2 days at 30 C, and the fungi after 5 days at 30 C. The fungi sporulated extensively. No spores, however, were seen in the *Bacillus subtilis* cultures. Repeated attempts to grow another actinomycete, *Streptomyces lavendulae*, in the nutrient broth glucose medium were unsuccessful.

TABLE 1

Constancy of the amino acid composition of microorganisms when cultivated repeatedly under identical growth conditions

ORGANISM	YIELD DRY WT PER LITER	NITROGEN	HISTIDINE	ARGININE	LYSINE	LEUCINE	ISOLEUCINE	VALINE	METHIONINE	THREONINE	PHENYLALANINE	TRYPTOPHANE
		Percentage of dry weight										
	grams											
Staphylococcus aureus												
Batch 1	0.295	10.75	0.72	2.3	5.2	3.4	2.8	2.4	0.81	2.0	1.84	0.23
Batch 2	0.228	11.23	0.84	2.1	5.6	3.4	2.9	2.9	0.91	2.2	2.3	0.35
Batch 3	0.335	11.21	0.81	2.0	5.4	3.4	2.8	2.5	0.86	2.2	2.1	0.25
Streptomyces griseus												
Batch 1	2.25	9.28	0.81	2.9	2.0	3.6	1.60	3.3	0.53	2.2	1.55	0.56
Batch 2	2.25	9.09	0.85	2.9	2.2	3.8	1.63	3.5	0.56	2.4	1.65	0.68
Batch 3	2.25	9.06	0.85	2.9	2.2	3.8	1.44	3.6	0.56	2.4	1.50	0.61
Aspergillus niger												
Batch 1	3.5	5.21	0.90	1.04	1.04	1.48	0.88	1.09	0.22	1.11	0.85	0.26
Batch 2	4.0	5.11	0.95	1.22	1.15	1.48	1.04	1.17	0.22	1.12	0.84	0.25
Batch 3	3.5	5.29	1.08	1.07	1.17	1.52	0.92	1.16	0.22	1.10	0.89	0.30

When the quantities of the amino acids are expressed as percentages of the dry weight of the cells, as in table 2, it is clear that marked differences exist between the bacteria and to a lesser extent between the two yeasts and the three fungi. Thus *E. coli* contains somewhat more isoleucine and threonine than *S. aureus* and approximately twice as much histidine, arginine, leucine, valine, methionine, and phenylalanine, and three times as much tryptophane. *S. aureus* exceeds *E. coli* only in having a slightly greater content of lysine. *B. subtilis* is intermediate between *S. aureus* and *E. coli* with respect to content of the ten amino acids. Also *Saccharomyces cerevisiae* has a higher content of histidine, leucine, isoleucine, methionine, threonine, phenylalanine, and tryptophane than *Rhodotorula rubra*, but less of arginine. For all of the amino acids, however, the yeasts differ by less than a factor of 2. Although *Rhizopus nigricans* and *Aspergillus niger* contain essentially the same quantities of the ten amino acids, *Penicillium notatum* has a higher content of histidine and of most of the other amino acids.

TABLE 2

Amino acid composition of representative microorganisms

ORGANISM	NITROGEN	HISTIDINE	ARGININE	LYSINE	LEUCINE	ISOLEUCINE	VALINE	METHIONINE	THREONINE	PHENYLALANINE	TRYPTOPHANE
	Percentage of dry weight										
Staphylococcus aureus	10.75	0.72	2.3	5.2	3.4	2.8	2.4	0.81	2.0	1.84	0.23
Escherichia coli	13.19	1.26	4.3	4.5	6.4	3.8	4.5	1.7	3.2	2.7	0.79
Bacillus subtilis	10.07	0.87	2.4	3.4	4.8	3.0	3.5	1.08	2.2	2.2	0.38
Streptomyces griseus	9.09	0.85	2.9	2.2	3.8	1.63	3.5	0.56	2.4	1.65	0.68
Saccharomyces cerevisiae	8.94	2.7	2.4	3.1	3.8	2.5	2.8	0.65	2.4	2.1	0.59
Rhodotorula rubra	8.95	1.99	3.7	3.0	3.3	2.1	2.5	0.53	1.79	1.72	0.45
Rhizopus nigricans	5.80	0.98	1.21	1.59	1.46	0.98	1.08	0.33	0.96	0.81	0.25
Aspergillus niger	5.21	0.90	1.04	1.04	1.48	0.88	1.09	0.22	1.11	0.85	0.26
Penicillium notatum	6.13	1.67	1.40	1.53	2.1	1.22	1.51	0.39	1.37	1.16	0.48

With the exception of histidine and tryptophane, the fungi contain generally less than half the quantitity of amino acids present in the bacteria, actinomycete, and yeasts. This is due largely, although not completely (see figure 1), to the lower protein content of the fungi as indicated by their considerably lower nitrogen content. There are no outstanding differences between the bacteria, actinomycetes, and yeasts as groups. The variations between them are no greater than those within the bacterial group itself except for methionine, which is present in appreciably greater quantity in the bacteria. In general, the amino acids in the various microorganisms can be classified roughly in the following decreasing order of abundancy: leucine > lysine > valine > arginine > isoleucine > threonine > phenylalanine > histidine > methionine > tryptophane. This sequence may vary slightly for any particular organism.

It is of more fundamental significance to compare the amino acid composition of the microorganisms on the basis of percentages of the amino acids in the protein of each organism, rather than as percentage of dry weight. Differences obtained by the latter method may merely reflect variations in the amount of protein in the different cells, as is largely the case when bacteria are compared to fungi, rather than to differences in the proteins themselves. Therefore, the data in table 2 have been recalculated in terms of percentage of protein (N × 6.25) and charted in figure 1. Data on wheat and beef liver obtained previously (Stokes *et al.*, 1945) are included for comparison.

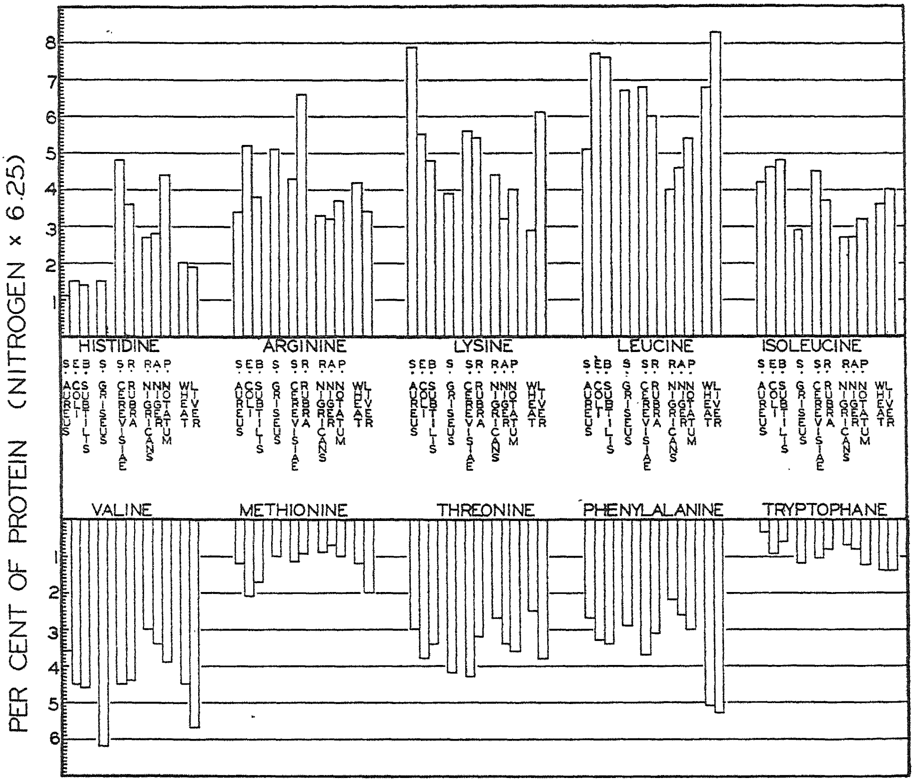

FIG. 1. THE AMINO ACID COMPOSITION OF REPRESENTATIVE MICROORGANISMS

Although the data represented in figure 1 is considerable, it covers too few organisms to permit any broad generalizations. With this limitation in mind, certain salient facts may be mentioned. The yeasts and fungi contain approximately 2 to 4 times (2.7 to 4.8 per cent) as much histidine as the bacteria (1.1 to 1.5 per cent) or the actinomycete (1.5 per cent). This is unusual since, except for tryptophane, the fungi contain less of the other amino acids than the bacteria. The bacteria and the actinomycete also contain somewhat less histidine than wheat or liver. Large variations occur within some of the groups. Thus *R. nigricans* contains 2.7 per cent histidine compared to 4.4 per cent in *P. notatum.*

The foregoing quantitative sequence for histidine changes somewhat in the case of arginine. The yeasts are highest with 4.3 to 6.7 per cent arginine, but the fungi contain the least amount of this amino acid, 3.2 to 3.7 per cent. The actinomycete and bacteria are intermediate between the yeasts and fungi. *R. rubra* is especially high in arginine (6.7 per cent). Differences between groups are not so great as with histidine. Variations within groups are evident. The bacteria and yeasts contain more lysine than the other groups. *S. aureus* has an especially high lysine content of 7.8 per cent. The differences between wheat and liver encompass all of the variations between and within the microbial groups. The fungi contain less leucine than any of the other microorganisms. The latter contain roughly similar quantities of leucine (6.0 to 7.7 per cent), which are comparable to those in wheat and liver, except for *S. aureus* which has a low value of 5.1 per cent. The bacteria are consistently higher in isoleucine than the remaining groups including the plant and animal proteins. This holds also for valine except that the actinomycete contains more of this amino acid than the bacteria. The amounts of methionine in all cells are much less than those of any other amino acid except tryptophane. The bacteria again lead all other microbial groups, and the fungi contain the least amounts of methionine. In contrast to the other amino acids, there are no marked differences in content of threonine between groups. Differences within groups, however, are evident. All of the microorganisms contain much less phenylalanine (2.2 to 3.7 per cent) than wheat or liver (5.1 to 5.3 per cent). Tryptophane is present in all of the groups in smaller amounts than any of the other amino acids. It is noteworthy that the bacteria, except *E. coli,* contain less tryptophane than the other microbial groups.

The general picture appears to be one in which variations in amino acid content within the microbial groups is frequently greater than those between groups. Although striking quantitative differences occur between organisms, the data, in general, emphasize the similarities rather than differences in the amino acid composition of microorganisms. Certainly no fundamental differences, in that some amino acids are present in one organism but not in another, as found among algae (Mazur and Clarke, 1938), were disclosed. There is, however, a consistent and probably significant trend in fungi toward lower contents of from 10 to 50 per cent of most of the amino acids compared to the contents in other microbial groups. This is compensated for to some extent by the higher histidine content of the fungi and presumably by higher contents of some of the other ten or more amino acids which occur in proteins and which were not determined in the present investigation. No such consistent trend is evident between the bacteria, actinomycete, and yeasts. Similarly, in agreement with comparable data of Camien *et al.* (1945) and Block and Bolling (1945), the microbial proteins do not appear to differ basically from the plant and animal proteins represented here by wheat and liver.

Mycelium and spores. The previous amino acid analyses of the fungi were made on cultures consisting of both vegetative cells (mycelium) and spores. To compare the amino acid composition of these two morphological components, and also of the mycelium before and after sporulation, *Aspergillus niger* was grown in the previously mentioned nutrient broth glucose medium. Twenty-five 2-L flasks each containing 400 ml of broth were inoculated with a spore suspension of *A. niger*. After stationary incubation at 30 C for 1 day, during which a white, thin, surface membrane of mycelium free of spores had formed, the growth from five flasks was collected, washed, and dried. The yield was 2.5 g. After 3 days, at which time a dense layer of black spores covered the entire surface of the fungal mat, the growths from two flasks were removed and thoroughly shaken and scrubbed in about a dozen changes of water to remove as many spores as possible. The yield was 730 mg of dry mycelium contaminated with only a few spores. The spent broth from the remaining 18 flasks was decanted and replaced twice, carefully, so as not to wash off the spores, with about 200 ml of water to wash the bottom of the mycelial pads free of medium. On the third replacement, the heavily sporulated mats were shaken thoroughly to separate the spores from the mycelium. After filtration through cotton gauze to remove pieces of detached mycelium, the spore suspension was centrifuged and the sedimented spores were washed twice with water and dried at 100 C. Considerable amounts of the water-soluble, dark brown pigment of the spores were lost during these operations as well as a large portion of the spores which adhered tenaciously to the mats. Microscopically, roughly 30 per cent of the spores were light brown, but the remainder were dark brown. Dried spores (3.2 g) were obtained that were almost completely free of mycelium.

TABLE 3

Amino acid content of mycelium and spores of Aspergillus niger

COMPONENT OF CULTURE	NITROGEN	HISTIDINE	ARGININE	LYSINE	LEUCINE	ISOLEUCINE	VALINE	METHIONINE	THREONINE	PHENYLALANINE	TRYPTOPHANE
	Percentage of dry weight										
Whole culture	5.21	0.90	1.04	1.04	1.48	0.88	1.09	0.22	1.11	0.85	0.26
Mycelium before sporulation	7.61	1.20	2.6	2.7	2.6	1.41	1.83	0.52	1.67	1.48	0.52
Mycelium after sporulation	4.99	1.53	1.40	1.19	1.30	0.70	0.95	0.22	0.93	0.77	0.31
Spores	5.54	0.54	1.19	1.33	1.75	0.99	1.29	0.23	1.27	1.01	0.33

Since the tough, chitinous, outer covering of the spore might prevent hydrolysis of the spore protoplasm, digestion with acid and alkali was carried out for 20 hours as well as for the customary 10-hour period. Microscopically, the undigested solids in the hydrolyzates consisted of cracked or broken spore shells and seemingly intact shells, although ruptures could have been hidden from view. However, since the same quantities of amino acids were found in the 10- and 20-hour hydrolyzates, it was considered that complete digestion of the spore proteins had been effected and that only the chitinous shells of the spores remained undigested. Table 3 contains the percentages of the ten amino acids in the mycelium before and after sporulation and in the spores. The values for the whole *Aspergillus niger* culture from table 2 are included for comparison.

The young mycelium before it has sporulated contains about 50 per cent more protein, as calculated from its higher nitrogen content, than is present after sporulation. Largely because of this, the unsporulated mycelium contains considerably more of practically all the amino acids measured than the sporulated mycelium. The only exception is histidine, which is somewhat higher in the sporulated mycelium. In contrast, there is only about 10 per cent difference between the nitrogen content of the sporulated mycelium and its spores and, therefore, correspondingly smaller differences in the amino acid content of these two components. However, there is three times as much histidine in the mycelium as in the spores. The differences in the quantities of amino acids in the mycelium before and after sporulation and in the spores are reduced but not eliminated when the amino acids are calculated as percentage of protein (figure 2) rather than as percentage of dry weight.

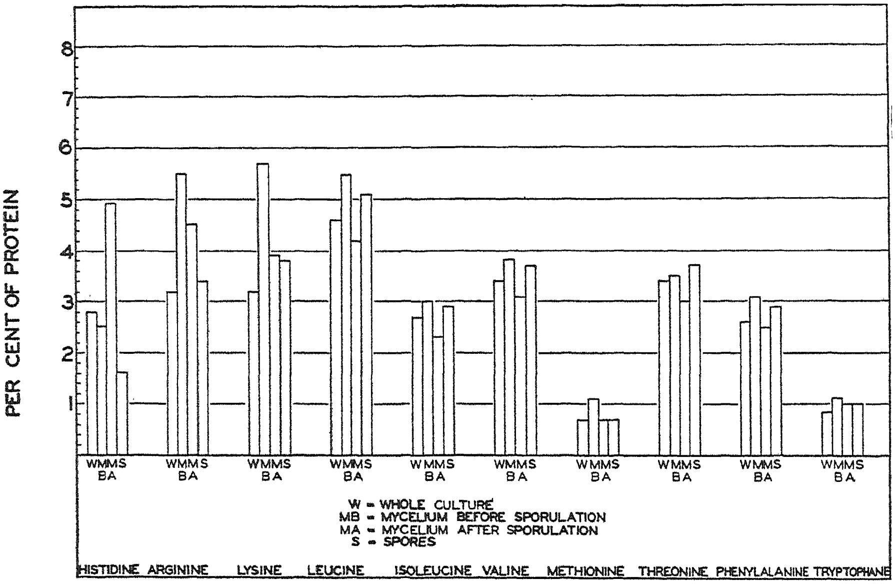

FIG. 2. THE AMINO ACIDS IN THE WHOLE CULTURE, MYCELIUM, AND SPORES OF ASPERGILLU NIGER

EFFECT OF MEDIUM, AGE, AND AERATION ON AMINO ACID COMPOSITION

The observations of Abderhalden and Rona (1905), Tamura (1913*a*), and Camien *et al.* (1945) indicate that the amino acid composition of microorganisms is independent of the composition of the growth medium. The data presented below indicate, however, that the amino acid content of microorganisms may vary with changes in growth medium and other environmental conditions.

Penicillium notatum. Amino acid analyses were made on cell material from the following cultures: (a) A tank culture was grown with aeration and agitation for 1 day in a medium consisting of 3 per cent lactose and 8 per cent by volume of corn steep liquor. The mold does not sporulate under these conditions. The washed and dried mycelium contained 8.93 per cent nitrogen. (b) Five-day-old, stationary flask cultures were grown in the lactose, corn steep liquor medium. Spores were present. The cellular material contained 5.20 per cent nitrogen. (c) Seven-day-old, stationary flask cultures were grown in a medium consisting of brown sugar, 20 g; corn steep liquor, 30 ml; $NaNO_3$, 3 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $ZnSO_4 \cdot 7H_2O$, 10 mg; and H_2O, 1 L. Numerous spores were present. The nitrogen content of the cellular material was 5.87 per cent. All media were adjusted to pH 7 prior to sterilization and inoculation, and the incubation temperature was 28 C. It may be noted that, as previously observed with *Aspergillus niger*, the unsporulated mycelium from the tank culture contained 50 per cent to 70 per cent more nitrogen than the stationary sporulated cultures. The amino acid values as percentage of protein are charted in figure 3. The data from figure 1 on *Penicillium notatum* grown in nutrient broth glucose medium is included for comparison.

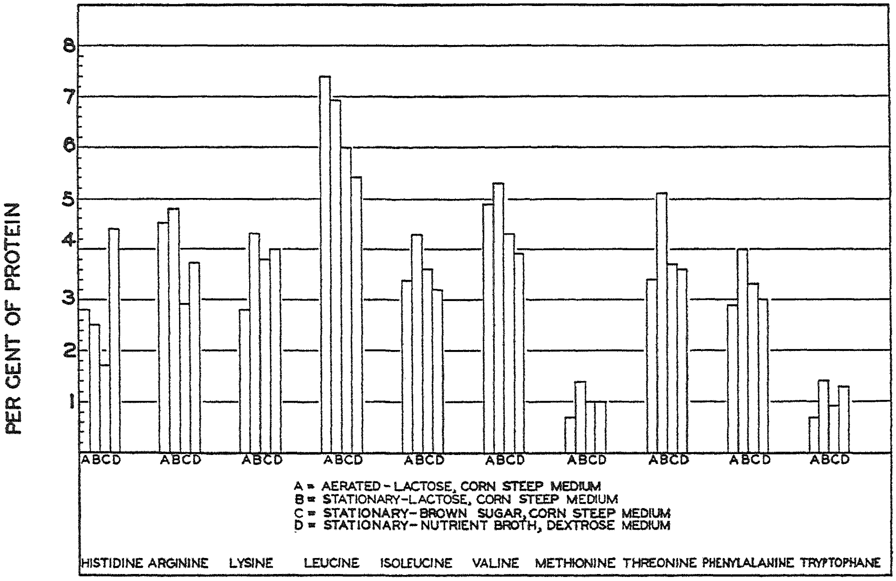

FIG. 3. EFFECT OF CULTURAL CONDITIONS ON THE AMINO ACID CONTENT OF PENICILLIUM NOTATUM

It is difficult to assess the effect of aeration versus nonaeration on amino acid composition because of the complicating factor of sporulation which occurred in the stationary but not in the submerged cultures. It is evident, however, that the aerated mycelium contains considerably less lysine, isoleucine, methionine, threonine, phenylalanine, and tryptophane than the unaerated cultures, but differs little from the latter in the other amino acids, histidine, arginine, leucine, and valine. The stationary cultures in the three different media which are comparable because sporulation had occurred in all of them show some interesting effects of medium on amino acid composition. Thus the cellular material from the nutrient broth medium contains roughly twice as much histidine as that from the other two media. The mold growth from the lactose medium, however, is considerably richer in most of the amino acids than that from the nutrient broth medium and to a somewhat lesser extent than the cellular material from the brown sugar medium. For example, such wide differences are encountered as 4.8 per cent arginine in the mycelium and spores from the lactose medium compared to 2.9 per cent in the fungal growth from the brown sugar medium. Similarly, 5.2 per cent threonine is present in the former compared to 3.6 per cent in the growth from nutrient broth.

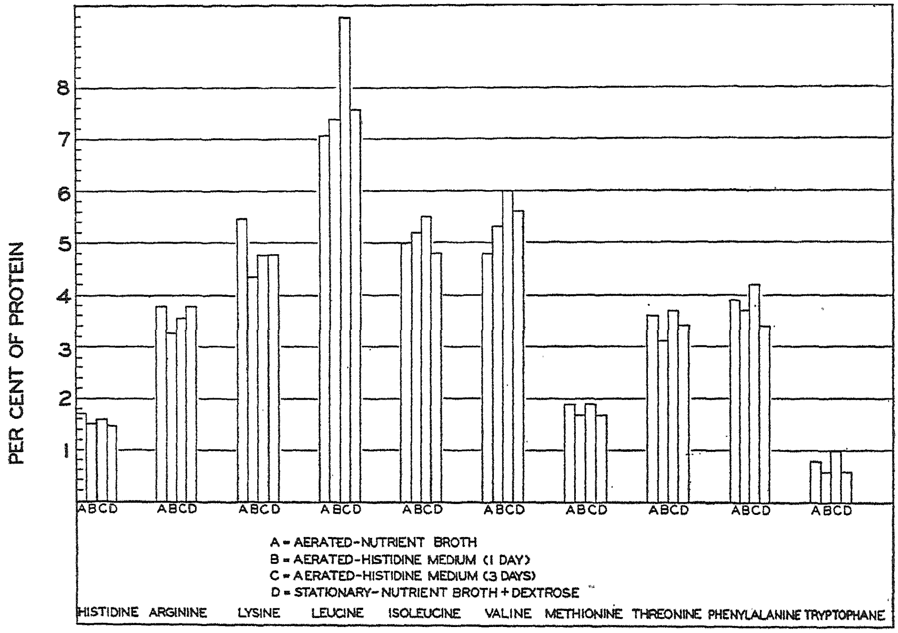

FIG. 4. EFFECT OF CULTURAL CONDITIONS ON THE AMINO ACID COMPOSITION OF BACILLUS SUBTILIS

Bacillus subtilis. Amino acid analyses were made on cell material from the following cultures: (a) Aerated nutrient broth cultures were incubated for 1 day. The yield was 0.3 g of dry cells per liter of medium with a nitrogen content of 10.39 per cent. (b) Stationary flask cultures were grown for 1 day in a synthetic medium containing histidine as the sole source of nitrogen. It consisted of glucose, 10 g; *l*(−)histidine, 1 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 10 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; $MnSO_4 \cdot 4H_2O$, 10 mg; and H_2O, 1 L. One-half gram of dry cells was obtained per liter of medium with a nitrogen value of 10.63 per cent. (c) Cultures were grown as in (b) except that the incubation period was extended to 3 days. Microscopically, considerable cellular debris from autolyzed bacteria was present in the 3-day-old histidine culture, but not in the 1-day-old culture. Six-tenths of a gram of dry cells per liter containing

11.22 per cent nitrogen were obtained. All media were adjusted to neutrality prior to sterilization, and the incubation temperature was 37 C. None of the cultures contained spores. This strain of *Bacillus subtilis* (ATTC 6633) can sporulate but does so only infrequently and unpredictably. The results of the amino acid analyses are plotted in figure 4 along with the data from figure 1 on *B. subtilis* grown in the nutrient broth glucose medium.

In contrast to the results with *Penicillium notatum* (figure 3), the amounts of most of the amino acids in *B. subtilis* are constant and, therefore, independent of the composition of the growth medium, aeration, and age of the culture. The many small differences obtained are within the experimental error of the analytical method. However, significantly more leucine, 8.8 per cent, was present in the cells from the 3-day-old histidine culture compared to 7.1 per cent to 7.6 per cent in the cells from the other media. Similarly, the bacteria from the aerated nutrient broth culture contained 5.5 per cent lysine compared to 4.4 per cent lysine in the cells from the 1-day-old histidine culture. In contrast, the former had 4.8 per cent valine compared to 6.0 per cent in the cells from the 3-day histidine culture. It is noteworthy that the cells grown with histidine as a nitrogen source did not contain significantly more of that amino acid than cells which had available complex nitrogenous substances, i.e., the meat extract and peptone of the nutrient broth.

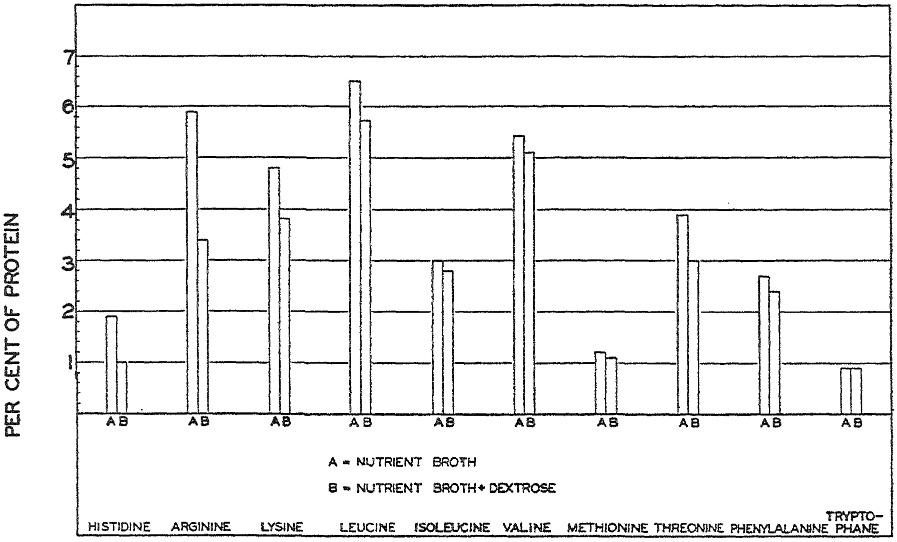

FIG. 5. EFFECT OF MEDIUM ON THE AMINO ACIDS OF STREPTOMYCES GRISEUS

Streptomyces griseus. The actinomycete was grown in nutrient broth and also in the same medium plus 1 per cent glucose. The media were distributed in 400-ml amounts in 2-L flasks. The inoculated flasks were placed on a shaking machine in a 30 C incubator for 2 days. The nutrient broth cultures yielded 1.6 g of dry cellular material per liter with a nitrogen value of 10.47 per cent, and the nutrient broth plus glucose medium yielded 1.1 g of cell substance with a nitrogen content of 10.48 per cent. It is clearly evident from figure 5 that the presence or absence of glucose in the medium has a striking effect on the amino acid composition of the cell material grown in such media. In the absence of glucose, *Streptomyces griseus* contained approximately twice as much histidine and arginine and considerably larger amounts of lysine, leucine, and threonine than did the cells grown with glucose. There were no significant differences between the two types of cells in the case of the remaining five amino acids.

The protein of a microbial cell is a complex mixture of a large variety of simple and conjugated proteins. Therefore, amino acid analyses of the total cell protein is, at best, a crude procedure which overlooks changes in the individual proteins unless they are accompanied by quantitative changes in the amino acids. Insofar as significant quantitative differences were obtained by varying environmental factors, it may be considered that these reflect important modifications in the character or distribution of the cellular proteins. These modifications may be in the enzymes of the cells rather than in their structural proteins, since they were generally not extensive and also because it might be expected that the nature of the cellular enzymes would vary with the available nutrients and other environmental factors.

TABLE 4

Comparison of the quantities of amino acids in Streptococcus faecalis and the amounts required for maximum growth

AMINO ACID	AMOUNT REQUIRED PER 10 ML MEDIUM FOR MAXIMUM GROWTH*	AMOUNT FOUND IN THE CELLS
	micrograms	*percentage of dry wt*
Lysine	200	5.0
Leucine	100	4.5
Isoleucine	100	4.1
Valine	100	3.9
Threonine	100	3.1
Arginine	80	3.0
Methionine	50	1.49
Histidine	40	1.04
Tryptophane	15	0.48

* Measured by titrating the lactic acid formed.

It is of interest that there is a positive correlation between the quantities of the different amino acids required for growth of *Streptococcus faecalis* in the synthetic medium previously described (Stokes *et al.*, 1945) and the amounts of these amino acids present in the dried cells taken from the same medium (table 4). This suggests that the amino acids of the medium are used primarily for synthesis of cellular proteins rather than being metabolized to smaller end products to supply the energy or other requirements of the cell.

SUMMARY

The quantities of ten amino acids, namely, histidine, arginine, lysine, leucine, isoleucine, valine, methionine, threonine, phenylalanine, and tryptophane, were

determined, microbiologically, in the acid or alkaline hydrolyzates of the dried cells of *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Streptomyces griseus*, *Saccharomyces cerevisiae*, *Rhodotorula rubra*, *Rhizopus nigricans*, *Aspergillus niger*, and *Penicillium notatum* grown under a variety of cultural conditions. It was found that the amino acid composition of an organism is, qualitatively and quantitatively, a stable and characteristic property of the cell under fixed conditions of growth. Although striking quantitative differences occur between microorganisms, the results, in general, emphasize the similarities rather than the differences in their amino acid composition. Certainly no fundamental differences in that some amino acids are present in one organism but not in another were encountered. The microbial proteins do not appear to differ materially from plant and animal proteins, represented by wheat and beef liver. Fungi contain 10 to 50 per cent less, per unit of protein, of most of the amino acids determined than do the other microbial groups. Mold mycelium prior to sporulation, compared to that after sporulation, contains considerably larger quantities of most of the amino acids largely because of its 50 per cent greater protein content. The mycelium and its spores, in general, have comparable amino acid contents. The quantities of individual amino acids in microorganisms may vary with the growth medium, aeration, and age of the cells.

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STUDIES ON LYOPHILED CULTURES:

LYOPHILE STORAGE OF CULTURES OF RHIZOBIUM LEGUMINOSARUM

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The preservation of bacterial cultures by various means has been the subject of much study, and the literature has been reviewed by Morton and Pulaski (1938). The lyophile method of preservation, which involves quick freezing and vacuum drying, is one of the most satisfactory methods developed. It has distinct advantages when cultures are to be shipped to distant places because the small containers, in contrast to ordinary test-tube cultures, can be shipped via air mail at relatively low cost and without danger of contamination. The lyophile method also eliminates the tedious process of transferring cultures.

There is ample evidence that many genera of bacteria can be preserved by the lyophile method, but little with respect to the nodule bacteria, though the successful drying of these organisms was done in the pioneer work of Rogers (1914). Appleman and Sears (1944) have discussed the possibility of using cultures preserved by the lyophile method as inoculants for legumes. Legume nodule bacteria lyophiled by the method of Flosdorf and Mudd (1935, 1936) were used in the following investigations, begun in 1939, to determine whether this type of storage causes marked changes in the longevity of the various groups of legume nodule bacteria or has unfavorable effects upon the nitrogen-fixing efficiency of these organisms.

Young cultures of *Rhizobium leguminosarum* grown on asparagus mannitol media and transferred at regular intervals were emulsified in sterile distilled water and 0.5-ml portions pipetted into sterile cotton-stoppered 4-mm pyrex tubes. After being frozen quickly in glycerol and dry ice, the tubes containing the cultures were attached to a vacuum pump by means of rubber tubing and evacuated. A flask of P_2O_5 was used to absorb moisture and hasten drying. When the cultures were dry, the tubes were sealed near the top by means of a pin-point blast burner while they were still evacuated.

After storage in cabinets at room temperatures for 3½ to 4 years, they were opened and tested for viability, purity, and ability to produce nodules on appropriate host plants.

Special precautions were taken on opening the tubes to avoid contamination. Each tube was marked with a file, placed in a beaker of 95 per cent alcohol for several minutes, and then removed, drained, broken at the file mark, flamed rapidly, and the contents poured into a tube of asparagus mannitol broth. The cultures were then incubated at 28 C until turbidity developed, after which transfers were made to tubes of litmus milk and agar slants. Transfers from agar stock cultures of each strain were also inoculated into broth and later tested in litmus milk to determine the purity of the agar stock culture, which had been transferred at intervals, and that of the lyophiled culture.

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In a preliminary study of lyophiled cultures of *Rhizobium leguminosarum*, isolations from a number of commercial inoculants as well as cultures from root nodules which had not been selected by single-cell isolation were used. Of the 20 cultures examined, 19 showed growth in broth and 18 gave nodulation under greenhouse conditions. The only viable culture that failed to give nodulation showed peptonization in litmus milk and, on microscopic examination, proved to be a sporeforming rod. The examination of transfers from the original agar stock culture demonstrated that it too was impure.

TABLE 1

Comparative tests of lyophiled and stock cultures of Rhizobium leguminosarum for the inoculation of legumes

PLANT	NUMBER OF CULTURES USED	TYPE OF PRESERVATION	NUMBER OF CULTURES PRODUCING NODULES	AVERAGE NUMBER OF PLANTS PER JAR	AVERAGE NUMBER OF NODULES PER PLANT
Alfalfa	6	lyophile	6	41.1	7.6
	6	stock	6	63.6	13.5
Korean lespedeza	3	lyophile	3	72.8	5.3
	3	stock	3	62.5	4.2
Cowpea	5	lyophile	4	1.8	51.1
	5	stock	4	2.8	37.3
Little marvel pea	3	lyophile	3	8.0	64.8
	3	stock	3	7.0	66.5
Illini soybean	5	lyophile	5	5.0	17.4
	5	stock	5	5.8	18.9
Vetch	3	lyophile	3	11.5	10.3
	3	stock	3	14.0	10.3
Crown vetch	3	lyophile	3	11.0	19.2
	3	stock	3	19.7	18.2
Medium red clover	6	lyophile	6	79.2	8.3
	6	stock	6	111.4	10.3

In order to determine whether lyophiling had affected nitrogen-fixing ability, a number of lyophiled cultures of the more common groups of *Rhizobium leguminosarum* were tested in comparison with transfers from corresponding stock cultures which had been maintained by the transfer method from those furnishing the original lyophiled material. Those included were alfalfa, lespedeza, cowpea, little marvel pea, soybean, vetch, crown vetch, and medium red clover.

When turbidity was noticed in the tube of broth inoculated with a lyophiled culture, one 0.2-ml sample was pipetted from this tube and another from the tube in which the corresponding culture was growing, and each sample was used to inoculate 6-gram portions of surface-sterilized seed. These portions of seed were planted in duplicate jars of sterile quartz sand. The fifth jar in each row of five was planted with uninoculated seed and used as a control. The sterile sand was saturated with sterile nutrient solution made up of all the necessary elements except nitrogen, and subsequent watering was done with sterile distilled water or dilute nutrient solution.

After 10 to 11 weeks the plants were washed free of sand and examined for the size and the color of the plants, as well as for the number and the placement of nodules. In all cases the plants grown as uninoculated controls had no nodules and exhibited symptoms of nitrogen starvation, being yellow to yellow-green in color and smaller than the plants grown from inoculated seed. On the other hand, plants grown from inoculated seed were vigorous, dark green, and well nodulated, whether from stock or lyophiled cultures (table 1). The fresh agar stock cultures were less effective in producing nodules on lespedeza and cowpeas and more effective on alfalfa and medium red clover than lyophiled legume nodule bacteria. On peas, soybeans, vetch, and crown vetch the two kinds of culture were equally effective.

SUMMARY

Legume nodule bacteria originally isolated from alfalfa, lespedeza, cowpea, pea, soybean, vetch, crown vetch, and clover host plants were lyophiled and preserved for periods of 3½ to 4 years, after which their ability to grow, nodulate host plants, and fix nitrogen was undiminished as compared to that of corresponding stock transfer cultures.

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ORGANIC GROWTH ESSENTIALS OF THE AEROBIC NONSULFUR PHOTOSYNTHETIC BACTERIA[1]

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Ever since the nonsulfur purple and brown bacteria were defined as a taxonomic entity—the *Athiorhodaceae*—it has been recognized that one of their distinctions from other photosynthetic bacteria was their failure to grow in inorganic media even in the presence of a suitable carbon-hydrogen-energy source ("substrate"); they had additional nutritional requirements which were met by inclusion in the medium of a small amount of yeast extract or peptone (van Niel, 1941, 1944). Despite this experimental imprecision, studies of these bacteria contributed data indispensable in erecting a satisfactory theory of the mechanism of photosynthesis (van Niel, 1941, 1944; Franck and Gaffron, 1941; Rabinowitch, 1945). The facultative aerobes in this group can grow aerobically in the dark—an indication of the manner in which the photosynthetic and nonphotosynthetic ways of life may have been linked phylogenetically. This fundamentally important type of evolutionary transition has been studied intensively from the nutritional standpoint only in algae, algal flagellates, and their colorless counterparts (Lwoff, 1944). It was evident from these considerations that the identification of the essential nutrients for *Athiorhodaceae* was of extraordinary interest from the standpoint of comparative cell physiology.

To summarize the results of the present study: of 124 aerobic isolates tested, 121 grew in synthetic media containing thiamine, biotin, *p*-aminobenzoic acid, nicotinic acid, or an appropriate combination of these. Different strains assigned by other criteria to the same species were remarkably similar in vitamin requirements, and each of the 5 species studied had a different pattern of vitamin requirements.

The technique for an investigation of unidentified growth factors should be rigorous enough to detect with certainty new vitamins of even greater ubiquity and potency than *p*-aminobenzoic acid, biotin, and folic acid. Procedures were developed which seemed adequate for the purpose and were convenient. As some of these methods may be unfamiliar in detail, an extended description follows.

To obtain large numbers of cells with a minimum of manipulation and a minimal carry-over of nutrients, and to exercise closer control over the purity of cultures, it was considered desirable to inoculate flask cultures from agar slants. All but 5 or 6 of the isolates grew well on moist slants, the greater number growing very heavily. Screw-capped tubes proved excellent for minimizing drying of the slants. After seeding, the plastic caps could be closed tightly without noticeable hindrance to growth. When water of syneresis was insufficient, a few drops of distilled water (likewise sterilized in screw-capped tubes) were added. It was found desirable to remove the liners from the caps in order to avoid unintentional sealing and consequent building up of pressure differences when the caps were screwed on loosely for sterilization. The experiments were conducted with 25-ml Erlenmeyer flasks containing 10 ml of medium and capped with 10-ml beakers; 50-ml flasks supported growth no better. Many flasks had their rims ground down on a fine emery wheel to enable the beakers to fit easily.

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[1] A preliminary report was presented before the New York City Branch of the Society of American Bacteriologists (J. Bact., **51**, 405, 1946).

[2] I am indebted to Miss Marjorie Jane Dean of Wilmington College, Wilmington, Ohio, and to Miss Frances E. Carey for aiding in this work and to Dr. Mortimer P. Starr of Brooklyn College, who, while a National Research Council fellow at the Hopkins Marine Station of Stanford University, forwarded the cultures. Thanks are also due to Professor C. B. van Niel for making the cultures available and for other encouragement.

Inoculation pipettes were sterilized by autoclaving in individual pyrex tubes plugged at both ends with cotton that had been sterilized repeatedly. Dry sterilization led to the separation of tarry breakdown products from the cotton. The autoclaved pipettes in their tubes were dried at temperatures below 80 C. By this procedure the pipettes were not subjected to contact with metal containers, with risk of toxicity and of interference with experiments on trace element requirements.

All media were autoclaved 10 minutes at 118 to 121 C. In preliminary trials, a disquieting number of air-borne contaminations were observed in the beaker-capped flask cultures. These contaminations were traced to the overrapid influx of air into flasks cooling after autoclaving and were eliminated by allowing the flasks after sterilization to cool in the autoclave over a period of at least 5 hours, with the exhaust valve kept shut. Then, if a vacuum was still present, air was admitted very gradually. Contaminations of agar slants were similarly minimized by allowing the melted agar to cool in the autoclave in a slanted position and by then screwing the caps on tightly as the tubes were removed.

A potential source of error was that resulting from inapparent bacterial growth in components of culture media supplied from stock solutions. Even a scarcely perceptible growth of microorganisms in a solution, followed by their death and lysis and by the restoration of clarity to the solution, might furnish a significant residuum of growth factors. It was inconvenient to weigh out every component of the medium for each experiment, and especially inconvenient for trace elements. Satisfactory preservation of highly putrescible solutions of yeast extract and peptones could be achieved by storing such solutions in glass-stoppered pyrex bottles, adding at least 1 per cent of a 1:1 mixture of redistilled CCl_4 and toluene, and keeping at 6 C. These solvents were completely removed on autoclaving. Constant vigilance had to be exercised to detect and prevent microbial spoilage of solutions. For instance, solutions in pyrex bottles of salts of boron and molybdenum acidified with HCl, but when kept at room temperature, eventually became contaminated despite the presence of a preservative. Subsequent experiments revealed that these salts had a favorable assortment of trace element impurities—perhaps the reason they were good substrates for the germination of food-laden bacterial and fungal spores. After extensive trials[3] it appeared likely that a 3:1 mixture of *n*-butyl chloride and CCl_4 formed a more effective volatile preservative.

The following simple inoculation procedure reduced carry-over effects to negligible proportions. A loopful of slant growth was suspended in 20 ml of the basal medium in a "dilution" flask, and each experimental flask received one drop of this supension. From time to time carry-over effects were estimated by suspending a loopful of culture from a flask culture into a fresh dilution flask and by inoculating a duplicate series of flasks. No significant carry-over effects were observed. It was sometimes necessary to seed a hundred or more flasks from one dilution flask, using a single pipette. The risk of contamination incurred by this eggs-in-one-basket procedure was effectively minimized by avoidance of the violent air currents generated by a flame. The flame was used only for sterilizing the wire loop; the preparation of the cell suspension and the subsequent pipetting proceeded with the flame turned off.

All cultures were grown at room temperature (22 to 30 C) under 40-watt tungsten lamps at a distance of 30 to 60 cm that was arranged to furnish fairly even illumination. The illumination did not appear critical except in respect to the danger of overheating the cultures, and appeared adequate for even relatively dense cultures. As many of the bacteria did not grow well above 31 C, overheating was a serious problem when large numbers of flasks were used at one time during the summer.

Agar slants. Many different media proved suitable for the maintenance of cultures. The substrate was adequately supplied as lactate, 0.2 to 0.4 per cent, or malic acid (natural or synthetic), 0.1 to 0.4 per cent; occasionally Na-acetate. $3H_2O$, 0.05 to 0.1 per cent, or Na-butyrate, 0.04 per cent, was added to malate and lactate media. Media not containing malate contained Na_3-citrate $.2H_2O$, 0.025 to 0.1 per cent, to ensure full availability of heavy metals and calcium. The unidentified requirements were adequately supplied as trypticase (Baltimore Biological Laboratories), 0.1 to 0.2 per cent, or thiopeptone (Wilson), 0.1 per cent. Yeast extract (Difco) was inhibitory to many strains; trypticase was non-inhibitory and permitted extremely good growth. The remainder of the medium consisted of agar, 1.5 per cent; small amounts of K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $(NH_4)_2HPO_4$, and Fe, 0.1 to 0.4 mg per cent; and Mn, 0.05 to 0.2 mg per cent. The pH was adjusted to 6.5 to 6.8. A few isolates designated "*Rhodovibrio*," which appeared to be microaerophilic, and a few isolates of *Rhodospirillum rubrum*, which, although uninhibited by air, seemed unusually sensitive to inhibitory substances or were exacting for other reasons, were grown on the same media rendered semisolid by decreasing the agar to 0.2 to 0.4 per cent.

The growth of slant cultures was usually heavy in 24 to 48 hours. They were then stored in the dark at 6 C. They remained satisfactorily viable for at least a month; indeed many strains grew appreciably during such storage. Cultures older than a month were not, however, used for inoculating experimental flasks. There was no obvious impairment of photosynthetic ability as a result of this treatment.

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[3] Bjerknes, Clara A., and Hutner, S. H. Presented before the New York City Branch of the Society of American Bacteriologists, April, 1946.

RESULTS

Of 17 isolates previously identified as *Rhodospirillum rubrum*, 15 required biotin; 2 did not grow in the synthetic medium. Among the isolates growing in synthetic media were the Esmarch, Muller, and Lister strains. The isolates not growing in synthetic media were those designated at no. 5 (E III 2.1.I.b)and no. 8 ("Giesberger").

All 34 isolates of *Rhodopseudomonas palustris* required *p*-aminobenzoic acid and grew readily in synthetic media. Isolates used by Gaffron were included in this collection.

TABLE 1

Basal medium for identification of vitamin requirements

	g		*mg*
K_2HPO_4	0.05	B	0.005
KH_2PO_4	0.05	Ca	0.5
$(NH_4)_2HPO_4$	0.08	Cu	0.001
$MgSO_4 \cdot 7H_2O$	0.02	Fe[*]	0.2
Lactate	0.3	Ga	0.001
Na-acetate$\cdot 3H_2O$	0.1	Mn[†]	0.1
Na_3-citrate$\cdot 2H_2O$	0.1	Zn	0.2

Distilled water to 100 ml

pH adjusted to 6.6 to 6.8 with NaOH

Vitamins supplied when necessary as follows: thiamine 0.1 mg, nicotinic acid 0.1 mg, *p*-aminobenzoic acid 0.01 mg, and biotin[‡] 0.4 μg per cent. Elements obtained as metals were dissolved in a small amount of HCl, HNO_3, or aqua regia.

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[*] Westinghouse high purity iron supplied through the courtesy of Mr. E. B. Ashcraft.

[†] Electrolytic manganese kindly furnished by the U. S. Bureau of Mines.

[‡] Gift of Merck and Co., Inc.

In the early experiments with lactate media, 14 of the 15 isolates of *Rhodopseudomonas capsulatus* required thiamine alone. The same batches of media had been successfully used in detecting the biotin and nicotinic acid requirements of *R. gelatinosa* and *R. spheroides.* On rechecking these early results with purer media containing synthetic malate, however, thiamine alone was inadequate for 2 of the 3 isolates tested; biotin plus nicotinic acid was also necessary. Further work is under way to determine in what manner the vitamin requirements of strains of this species vary with the other constituents of the medium. The strain that in many experiments did not grow in synthetic media was the one designated as no. 26 ("*Streptococcus varians* C10").

All 20 isolates of *Rhodopseudomonas gelatinosa* required biotin + thiamine, and all 17 isolates of *R. spheroides* required biotin + thiamine + nicotinic acid. Among these isolates was one designated as "*Streptococcus varians* (original)" and two designated as "*Phaeomonas.*"

It is evident that success was obtained in determining the requirements of the aerobic strains employed by other workers in studies of photosynthesis.

Twenty-one miscellaneous unclassified isolates were assigned to one or another of the 5 species on the basis of vitamin requirements; a superficial examination showed no obvious discrepancies between the new taxonomic criterion represented by the growth factor requirements and the original criteria for these species. The diagnostic characters employed by van Niel (1944) to delimit these species were morphological (shape and arrangement of cells, color, and mucus formation, all at different pH's), and biochemical (liquefaction of gelatin and utilization of various oxidation substrates). The results are summarized in table 2.

TABLE 2

Vitamin requirements of the aerobic nonsulfur photosynthetic bacteria

	BIOTIN	*p*-AMINOBENZOIC ACID	THIAMINE	THIAMINE + BIOTIN	THIAMINE + BIOTIN + NICOTINIC ACID
Rhodospirillum rubrum	+				
Rhodopseudomonas palustris		+			
Rhodopseudomonas capsulatus			+*		+*
Rhodopseudomonas gelatinosa				+	
Rhodopseudomonas spheroides					+

* Some strains.

DISCUSSION

The uniformity of the vitamin requirements suggested that there might have been some duplication of strains. There was, however, considerable color and morphological variation among isolates within each species—a good indication of heterogeneity. Also many of the isolates were obtained from different localities and by different enrichment procedures. Most of the isolates studied here were obtained by Foster (1944), who used a variety of alcohols as enrichment substrates. In keeping with the taxonomic scheme proposed by van Niel (1944), *Rhodopseudomonas palustris* and *Rhodospirillum rubrum* thus form two distinct and rather homogeneous species, standing apart from each other and from the group represented by the rather similar *Rhodopseudomonas capsulatus*, *R. gelatinosa*, and *R. spheroides*. It is remarkable that no isolates were found without vitamin requirements, and that each of the 5 species should have a different vitamin requirement, considering how few growth factors were involved. The results thus furnish a neat example of how nutritional data may support taxonomic surmises based on other biochemical and morphological criteria. It will be of interest to determine whether the vitamin requirements are the same for cells grown in the dark, or in anaerobiosis. The problems of the nutrition of the obligate anaerobes and of the more exacting aerobic strains remain for the future.

The absence of any amino acid requirement, despite the presence of so many vitamin requirements, is an unusual state of affairs in microbiology. The stimulating effect of protein hydrolyzates, noted earlier (Hutner, 1944), disappeared when basal media were developed that contained good substrates and adequate amounts of trace elements.

It proved very difficult to duplicate in synthetic media the favorable effects of amino acids and protein hydrolyzates. The conclusion became inescapable after very many experiments that the limiting factors for growth, in the absence of compounds of biological origin, were likely to be essential elements as yet unidentified but occurring as extraordinarily potent impurities in the usual cp grades of trace elements.

Trace element requirements. Paradoxically, organisms with simple growth requirements present difficulties perhaps not fully appreciated by workers whose principal experience has been with forms having complicated organic requirements. Many compounds of biological origin commonly used in culture media, particularly those with metal-complex-forming groups, are heavily contaminated with essential trace elements; asparagine, glutamate, and sugars are noteworthy in this respect. Nearly all the photosynthetic bacteria here studied could be grown in media containing as sole organic constituents synthetic vitamins contributing an insignificant amount of trace elements, plus carbon-hydrogen-energy sources in the form of fatty acids or alcohols rendered metal-free by distillation. Hence in replacing biological materials such as yeast extract, peptones, natural amino acids, and protein hydrolyzates with purified synthetic compounds, it became necessary to make a special effort to provide adequate amounts of the trace elements required. The success of the medium described in table 1 was shown by later experiments to depend on some largely unforeseen factors:

(1) The *lactate* (prepated by neutralizing reagent lactic acid with ordinary reagent grade NaOH) was heavily contaminated with favorable trace elements. The source of this contamination was probably not only attributable to the biological origin of the lactic acid, but also to the calcium or zinc salts through which the lactic acid was purified and to the NaOH used for neutralization. Commercial salts of calcium and zinc, and ordinary NaOH appeared to be good carriers of essential trace elements. Lactate was utilized with a readiness unsurpassed by any other compound tested and, unlike the fatty acids, was completely devoid of inhibitory properties, even in relatively high concentrations (0.3 to 0.5 per cent) and through a wide pH range (6.3 to >8.5).

(2) The *trace element supplement* supplied the elements known or suspected to be essential in amounts clearly in excess of the true quantitative requirements for these elements. Later work with purified media indicated that these excessive concentrations of heavy metals were really necessary to provide adequate amounts of the essential but unidentified elements with which they were contaminated. The iron, zinc, and manganese were especially favorable, and could be increased manyfold without toxicity and with some betterment of growth as long as they did not give rise to precipitates; in fact they appeared to protect the bacteria against toxic heavy elements. This effect was shared to a greater or lesser extent by samples of several other elements, among them cobalt, vanadium,

molybdenum, nickel, iridium, and rhenium. Gallium and scandium and the usual trace elements were not limiting factors in these experiments.

With the increasing use of glass, stainless steel, and "de-ionized" water in the chemical industry, it is likely that in the future the chemicals used by the microbiologist will be much purer than those now prevailing. Hence the problem of trace element requirements will become more troublesome until much more information is gained about these elements. Since there appear to be great variations in the amount of essential trace elements fortuitously present in reagent grade chemicals, different laboratories studying the same organisms grown in simple media are likely to have difficulty in reproducing one another's results. A case in point is provided by *Azotobacter*, as reported by Burk and Burris (1941). Hence, in order to enhance the reproducibility of this work, great attention had to be paid to the trace element requirements. These studies are continuing. The necessity of further information in this direction was emphasized by Emerson and Lewis (1939), who found that the efficiency of photosynthesis in *Chlorella* was directly dependent on the supply of essential but poorly identified trace elements.

A potential source of error remaining to be evaluated was that forthcoming from the presence in the medium of citrate—a biological product. The citrate filled an indispensable function; by forming soluble co-ordinate complexes with essential metallic elements, it kept them from becoming unavailable by precipitation as highly insoluble phosphates and hydroxides. This need is not usually obvious when the complex media are used, as certain amino acids are efficient compex-formers (Johnson, 1943; Smythe and Schmidt, 1930). The need for complex-formers of the citrate type was intensified for the nonsulfur photosynthetic bacteria by the high calcium requirement exhibited by many of them, the absence of amino acids from the media, and the alkalinization of the media when they were grown on media containing utilizable organic acids. Fortunately, synthetic malic acid, a fairly good complex-former, was available and was well tolerated by the bacteria in experiments to determine whether the citrate was contaminated by any vitamins. Tests on 5 isolates of each of the 5 species yielded with malate the same results as were earlier found with lactate-citrate media, except for the instance already mentioned of certain isolates of *R. capsulatus*. At any rate, the citrate did not harbor any vitamins needed by the bacteria.

The calcium requirement. There was considerable variation in the calcium requirement from one strain to another. For some it was indispensable, e.g., certain strains of *Rhodopseudomonas capsulatus* and *Rhodospirillum rubrum;* yet for others it was not clearly demonstrable, as with certain strains of *R. palustris.* No calcium requirement was noted for *Protaminobacter albus* and *Chlorella* grown in the same media. Yet many quantitative experiments demonstrated that, when a calcium requirement was demonstrable at all, it was rather high, about 0.5 mg per cent. It was eventually realized that in order to judge the genuineness of the calcium requirement many factors had first to be evaluated; among the foremost, the impurities in calcium. At least 20 elements tend to be coprecipitated with calcium, and, as commercial calcium is itself in the last analysis the product of a biological enrichment from sea water, there is ample opportunity for contamination to occur; indeed certain rare earth elements are detectable in all calcium compounds (Sandell, 1944). A similar problem had been noted for *Chlorella:* Emerson and Lewis (1939) noted that photosynthesis was higher in media containing calcium carbonate, yet Trelease and Selsam (1939) obtained excellent growth in the absence of calcium. The disputed role of calcium in the metabolism of *Azotobacter* represents another parallel problem.

It may be gathered from the foregoing discussion that the identification of the organic requirements for the nonsulfur bacteria is but a prelude to the attack on the far more difficult problem of the inorganic requirements for growth and, more narrowly, for photosynthesis. These bacteria are, as pointed out by Foster and by van Niel, excellent subjects for such studies. Their rapid, heavy growth at room temperature, their utilization of distillable organic substrates, and their indifference to aeration in the presence of light and suitable substrates—all contribute to this suitability. In the later phases of this work, growth in synthetic media equaled that in media containing bacteriological peptones or yeast extract, but this goal has not yet been achieved with certain more highly purified synthetic media—an indication, if one were needed, that much remains to be done before the optimal conditions for growth of even the least exacting organism can be accurately defined.

SUMMARY AND CONCLUSIONS

Investigation of 124 isolates of aerobic nonsulfur purple and brown bacteria revealed that *Rhodospirillum rubrum* required biotin; *Rhodopseudomonas palustris*, *p*-aminobenzoic acid; *Rhodopseudomonas capsulatus*, thiamine and, in certain media, biotin + nicotinic acid in addition to thiamine; *R. gelatinosa* thiamine + biotin, and *R. spheroides* thiamine + biotin + nicotinic acid. One strain of *R. capsulatus* and 2 strains of *Rhodospirillum rubrum* appeared to have additional requirements aside from the carbon-hydrogen-energy source.

By the provision of suitable trace elements it was possible to grow the bacteria in media containing no organic constituents of biological origin. Some strains had an ostensible requirement for calcium.

The necessity of a better knowledge of inorganic requirements was emphasized.

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STUDIES ON THE NUTRITIONAL REQUIREMENTS OF STREPTOMYCES GRISEUS FOR THE FORMATION OF STREPTOMYCIN

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In view of the marked activity of streptomycin *in vitro* against a number of species of organisms relatively resistant to penicillin, such as *Escherichia coli* (Schatz, Bugie, and Waksman, 1944), certain of the *Salmonella* (Robinson, Smith, and Graessle, 1944), *Klebsiella pneumoniae* (Donovick, Hamre, Kavanagh, and Rake, 1945), and *Mycobacterium tuberculosis* (Schatz and Waksman, 1944), as well as its *in vivo* therapeutic behavior against such infecting agents (Jones, Metzger, Schatz, and Waksman, 1944; Robinson, Smith, and Graessle, 1944), investigations of this antibiotic as well as of the characteristics and growth requirements of the causative organism, *Streptomyces griseus*, are now being vigorously investigated in many laboratories. Chemical studies on streptomycin have already resulted in the preparation of crystalline derivatives (Fried and Wintersteiner, 1945; Kuehl, Peck, Walti, and Folkers, 1945).

Schatz, Bugie, and Waksman (1944) state that the production of streptomycin by *Streptomyces griseus* requires in the culture medium the presence of a specific growth-promoting substance supplied by beef extract or corn steep liquor. The medium recommended contained peptone, beef extract, glucose, and sodium chloride. We have studied various other materials as sources of nutrition for *Streptomyces griseus* and have found that it is possible to devise media including neither beef extract nor corn steep liquor that yield as much as 250 units[1] of streptomycin per ml, and from which streptomycin is recovered more readily in a purified state. The latter advantage arises because certain of the basic constituents of beef extract are concentrated in a manner similar to that of streptomycin and are found as impurities in the end product. The present paper deals chiefly with media employing soybean meal as the source of nitrogen.

The experiments described below were all conducted in a uniform fashion. A dilute suspension of *S. griseus* spores was prepared by suspending in distilled water the surface growth of this organism grown on Krainsky's asparagine glucose agar. Since the spores wet with great difficulty, the suspension was shaken with glass beads for half an hour. The resultant even suspension was stored at 4 C and was used for some months to inoculate the various media tested.

The media to be tested were dispensed in 200-ml amounts in the earlier experiments, and later in 100-ml amounts in 500-ml Erlenmeyer flasks. After being autoclaved, each flask was inoculated with 0.5 ml of spore suspension. The flasks were then incubated at 24 C on a shaking apparatus oscillating approximately 100 strokes per minute.

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[1] This is an average peak figure for medium no. 8. Individual shake flasks have on occasion been found to contain more than 350 units per ml of broth.

From the third through the sixth or seventh days of incubation, samples were taken daily for pH determination and streptomycin assay. The samples were clarified by centrifugation; the supernatant fluids were removed and heated in a boiling water bath for two minutes and assayed by the 2-ml broth dilution method (Donovick, Hamre, Kavanagh, and Rake, 1945). The constituents of the media employed and the streptomycin concentrations obtained in the various broths are shown in table 1 and figure 1. These data represent a summary of the results of many replicate experiments.

TABLE 1

Streptomycin production in various media

MEDIUM NO.	CONSTITUENTS*				NO. OF REPLICATE FLASKS	VOL. OF MEDIUM PER FLASK		DAYS OF INCUBATION				
	Soybean meal†	Glucose	Beef extract	Sodium chloride				3	4	5	6	7
		%	%	%		*ml*						
1	1.5	1.0			2	100	pH	6.8	7.0	6.9	7.5	7.8
							u/ml‡	1.6		5.7	5.7	9.8
2§	1.5	1.0	0.5	0.5	4	200	pH	7.0	7.1	7.4	7.2	7.4
							u/ml	6.1	10.7	27.2	37.4	73.2
3	1.5	1.0	0.1		3	100	pH	7.3	7.8	8.2	8.3	8.5
							u/ml	37.1	44.1	96.2	121.0	114.0
4	1.5	1.0	0.1	0.5	6	100	pH	7.1	7.7	8.2	8.4	
							u/ml	123.5	147.0	146.0	158.0	
5	1.5	1.0	0.2	0.5	5	100	pH	6.8	7.7	8.2		
							u/ml	69.2	156.0	160.0		
6§	1.5	1.0	0.5	0.5	7	100	pH	7.2	7.9	8.3	8.6	
							u/ml	41.5	100.0	168.0	181.0	
7	1.5	1.0		0.5	16	100	pH	7.1	7.6	8.1	8.3	..
							u/ml	120.5	170.0	187.5	212.0	
8	1.0	1.0		0.5	6	100	pH	7.0	7.1	7.4	7.9	8.3
							u/ml	129.0	148.0	201.0	236.0	237.0

* Made up in distilled water.

† The soybean meal employed contained from 41 to 44 per cent protein.

‡ Units streptomycin per ml broth.

§ Media "2" and "6" were the same except that "2" was dispensed in 200 ml per flask while "6" was in 100-ml amounts.

Under the conditions employed in these studies the volume of medium per flask was extremely important. For example, in medium no. 2 the average flask yielded in the broth only 73.2 units of streptomycin per ml in 7 days, rising to 140 units per ml at a later date. When only 100 ml of the same medium was employed per flask (see no. 6), the average flask contained 181 units per ml of broth on the seventh day. Consequently, in most of the experiments only 100 ml of medium per 500-ml Erlenmeyer flasks was employed.

If the media are considered in the ascending order of activity obtained in the broth, it will be noted that a medium consisting of only soybean meal and glucose (no. 1) is distinctly deficient, yielding less than 10 u per ml in 7 days. The addition of 0.1 per cent beef extract but no sodium chloride (no. 3) improves the medium considerably but is only approximately half as effective as adding sodium chloride and no beef extract to a soybean glucose preparation (nos. 7 and 8). It would appear from these results that beef extract may supply certain necessary salts but is otherwise not required when soybean meal is employed. In fact, when media 4, 5, and 6 are compared with 7 and 8 it appears that the addition of beef extract to a soybean meal, glucose, sodium chloride mixture is somewhat detrimental to the rise in streptomycin content in the broth. We have routinely observed that the rise in activity in a culture of *S. griseus* is greatest during or shortly after sporulation has begun. Thus, whereas mycelial growth is excellent in the presence of beef extract, sporulation is delayed. This may account for the lower streptomycin yields obtained in broths nos. 4, 5, and 6.

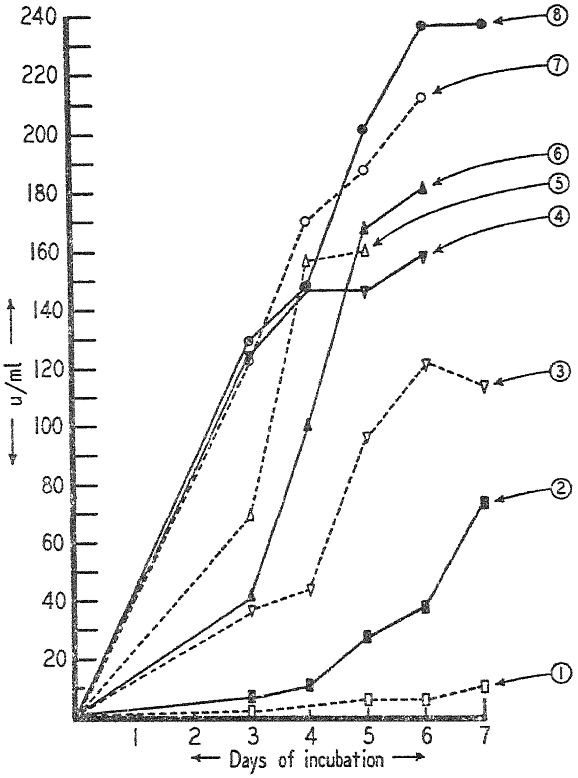

FIG. 1. STREPTOMYCIN PRODUCTION IN VARIOUS MEDIA

Preliminary studies have shown that substitution of the sulfate ion for the chloride ion in the salt added to soybean medium causes no appreciable change in the streptomycin concentration obtained in the broth. On the other hand, substitution of magnesium for the sodium ion gave lower streptomycin concentrations.

SUMMARY

The volume of medium in shake-flask cultures of *Streptomyces griseus* plays an important role in the concentration of streptomycin obtained in the broth.

Beef extract is not required for streptomycin production in a medium containing soybean meal, glucose, and sodium chloride. In fact, the addition of beef extract to such a medium in shake flasks delays somewhat the production of this antibiotic.

It is necessary to add an inorganic salt, e.g., sodium chloride, to soybean meal media for streptomycin production.

Preliminary studies indicate that sodium sulfate may be substituted for sodium chloride, but the substitution of magnesium chloride for either of these two electrolytes gives lower streptomycin yields.

Beef extract may supply a certain amount of the salt required in a soybean meal medium.

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PROTEINASE PRODUCTION BY BACILLUS SUBTILIS[1]

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In a study on the proteinases of food spoilage organisms it was observed that *Bacillus subtilis* failed to produce substantial amounts of proteinase in aerated nutrient broths prepared from Difco peptone and beef extract and from Difco dehydrated nutrient broth but that the organism showed slightly increased growth and good proteinase production when grown in nutrient broth containing Armour or Witte products. The deficiency was traced to the ash constituents in the latter nutriments and was found to be due, at least in part, to a

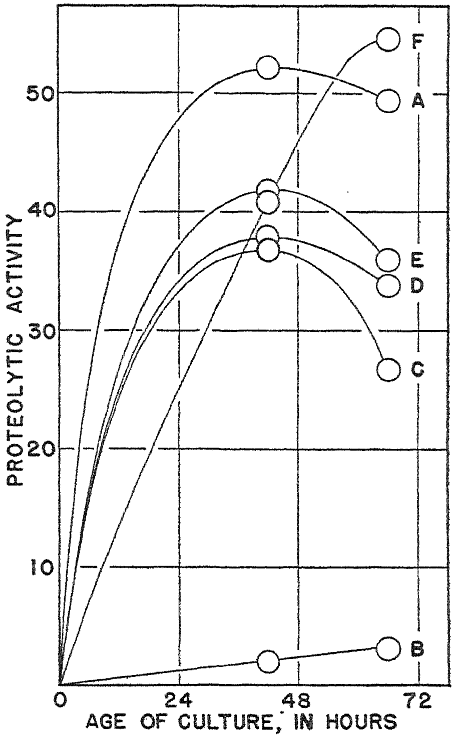

FIG. 1. EFFECT OF MN AND OTHER TRACE ELEMENTS ON PROTEINASE PRODUCTION

Digestion of casein in 5 ml of buffered 1% solution by 2 ml cell-free culture liquid acting 100 minutes at 30 C. Proteolytic activity measured as differences in percentage of transmittance of sulfosalicylic-acid-treated samples in Coleman Universal Spectrophotometer. *A*, Armour broth; *B*, Difco broth; *C*, Difco broth + Mn; *D*, Difco broth + Mn, Zn; *E*, Difco broth + Mn, Cu, Zn, B; *F*, Difco broth + Mn, Zn, Cu, B, Ti, Ni, Co, Mo, Br, I.

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[1] The subject matter of this paper has been studied in co-operation with the Quartermaster Corps Committee on Food Research.

lack of manganous ion (figure 1). Substantial proteinase formation may be obtained in Difco broth merely by adding 0.05 to 50 mg of $MnSO_4 \cdot H_2O$ per liter. The optimal concentration of added manganous salt appears to be approximately 5 mg per L. When manganous sulfate was added in comparable quantities to poorly proteolytic culture liquid at the end of the growth period, no increase in proteinase activity resulted. Therefore, the added manganese appears to function during the elaboration of the enzyme and not merely as an activator of the proteinase system.

Elements which do not replace the maganese effect are zinc, copper, iron, boron, magnesium, and calcium. Proteinase production seemed slightly greater, however, when a mixture of $MnSO_4 \cdot H_2O$ and $ZnSO_4 \cdot 7H_2O$ were employed instead of $MnSO_4 \cdot H_2O$ alone. It was still further improved upon addition of various combinations of the elements Mn, Zn, Cu, B, Ti, Ni, Co, Mo, Br, and I, now being used by Professor C. R. Johnson in studies on plant nutrition (figure 1). The desirability of Mn (and also Fe) in culture media intended for the production of bacterial proteinases by *Bacillus subtilis* has previously been indicated (Wallerstein, 1939; Boidin and Effront, 1930). However, Haines (1931) described protease formation in synthetic media in the absence of added Mn and Fe.

When cultures are constantly agitated, the proteinase content of the centrifuged broth is maximal within 2 or 3 days. Stationary cultures require more than 6 days for maximum proteinase accumulation, and less total proteolytic activity is obtained. The rapid growth and increased crop of highly aerobic organisms obtained in aerated cultures may call attention to nutritional deficiencies in culture media that appear adequate for stationary growth.

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A NEW LABORATORY FERMENTER FOR YEAST PRODUCTION INVESTIGATIONS

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Previously described laboratory yeast fermenters have not proved entirely satisfactory, chiefly because it has been extremely difficult if not impossible to obtain a sufficiently fine and uniform dispersion of air by means of sintered glass, porous ceramic discs or tubes, canvas bags, or similar devices commonly used (Colonial Food Yeast Limited, 1944; Fink *et al.*, 1941; Nolte *et al.*, 1942; Pavcek *et al.*, 1937; Peterson *et al.*, 1945; Stubbs, Noble, and Lewis, 1944; Willkie and Kolachov, 1942). Foaming may also become very troublesome, especially if high aeration rates are used. Most investigators have apparently focused their attention primarily upon total yields (Fink *et al.*, 1941; Peterson *et al.*, 1945) or upon vitamin content (Pavcek *et al.*, 1937; Scheunert *et al.*, 1939), and it has generally been assumed that aeration was adequate at all times. The importance of aeration has, however, been emphasized in a general review of the subject (De Becze and Liebmann, 1944).

Observations made in this laboratory[2] in connection with the development of a process for utilizing fruit wastes for yeast production led to the tentative conclusion that the attainment of high yeast cell concentrations in the culture liquid is dependent upon the effectiveness of aeration. A new laboratory fermenter was developed to test the validity of this conclusion, and it was found possible, by improvement of aeration, to obtain cell concentrations well in excess of those heretofore considered as the maximum and at the same time to maintain a normal propagation rate. This paper describes the new fermenter and presents some of the preliminary results obtained.

DESCRIPTION OF FERMENTER

The fermenter vessel consists of a standard section of pyrex pipe approximately 30 inches long and 3 inches inside diameter. The flanged ends of the glass pipe are fitted with gasketed metal covers, preferably constructed of stainless steel. The bottom cover provides an opening for the introduction of air and another for the withdrawal of samples. The top provides for the exit of air and for the introduction of supplemental nutrients.

The fermenter is fitted with a motor-driven stirrer. The stirring shaft and baffle assembly, constructed of stainless steel, is essentially a unit, and forms an integral part of the top cover. This feature minimizes alignment difficulties in the operation of the stirrer. The baffles, which consist of three equidistant

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[1] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

[2] Unpublished data obtained by I. C. Feustel and J. H. Thompson of this laboratory.

$\frac{1}{2}$-inch strips bent in a right angular form, are fastened directly to the top cover and are held rigidly in place by two supports that also serve as guide bearings for the stirring shaft. One of these supports is placed at the bottom end and the other approximately at the midpoint of the baffle assembly. A propeller ($2\frac{5}{8}$ inches in diameter) for dispersing air is mounted on the stirring shaft immediately below the bottom guide bearing. Three additional propellers ($2\frac{1}{8}$ inches in diameter) for aiding in air dispersion and mixing of the fermenter contents, are mounted at appropriate space intervals on the shaft. The design of the propellers seems relatively unimportant, but the stirring must be at high speed in order to accomplish the desired degree of air dispersion. A foam breaker is mounted on the shaft near the top of the fermenter and consists of four rectangular bars, each bent to an angle of about 45 degrees at the junction with the shaft but kept parallel to the plane of the culture liquid surface. This device not only breaks the foam of yeast cultures very effectively but also aids in mixing. The motor (1,550 rpm) is supported on the top cover and is held in place by means of clamps. It is connected to the shaft through a flexible coupling.

The entire fermenter assembly may be taken apart readily for cleaning, replacement or adjustment of parts, etc., or it may be disconnected from the motor and sterilized intact. The operating capacity of the fermenter ranges from approximately 500 to 2,000 ml.

EXPERIMENTAL PROCEDURE

Cultures of bakers' yeast, *Saccharomyces cerevisiae*, and *Torulopsis utilis* (Northern Regional Research Laboratory no. Y-900) were used for investigating the performance of the new fermenter. The procedures used for preparing inocula and media and for the conduct of propagation experiments were essentially similar to those previously described (Stubbs, Noble, and Lewis, 1944).

Yeast volume was used as a measure of the concentration of yeast cells in the culture liquid and as a means of estimating the growth or propagation rate. It was determined by centrifuging culture liquid in 15-ml graduated tubes, in duplicate, 4 minutes at 2,480 times gravity and was recorded as milliliters of yeast. The dry weight of the yeast was determined by carefully pouring off the supernatant liquid, resuspending the yeast in distilled water, and again centrifuging. The washing was repeated, and the centrifuged yeast was finally dried to constant weight in an oven. The average dry weight of yeast per ml of yeast volume was 0.22 g for *S. cerevisiae* and 0.20 for *T. utilis*.

The propagation or growth rate as used in this report is the percentage increase in the dry weight of yeast during 1 hour of growth. It is calculated from the difference in total yeast present in a culture at the end of any given hour as compared with the total present at the beginning of the hour. The yeast volume readings are used to estimate the respective weights of dry yeast. The calculated propagation rates are subject to some error because of inherent errors in yeast volume determinations, but the values provide a convenient means of appraising the progress and the final results of an experiment. Such values are probably as accurate as those obtained from actual cell counts.

Operation of fermenter. The initial nutrient solution (500 to 1,000 ml) which was prepared by diluting strong wort and which contained approximately 0.5 per cent sugar, together with a measured quantity of yeast inoculum, was placed in the fermenter through the funnel at the top. Stirring and aeration were started immediately.

The pH of the culture was determined every 15 or 30 minutes and was maintained at 5.0 to 5.5 by the addition of ammonia. Wort was added dropwise into the fermenter from a separatory funnel at a rate estimated to be sufficient for maintaining maximum growth of yeast during the progress of the experiment. Yeast volume readings were made every hour. The temperature was held at 30 C by means of a water bath in which the lower part of the fermenter was immersed.

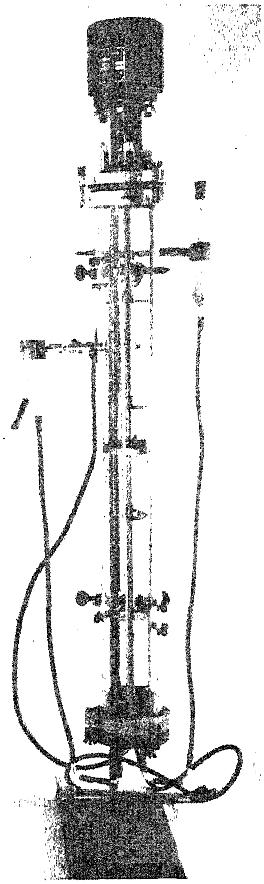

FIG. 1. NEW LABORATORY FERMENTER

After exploring a wide range, the rate of aeration adopted for routine use was approximately 1 liter of air per minute for each 10 grams of yeast (dry weight) contained in the fermenter. A wet-test gas meter was used to measure the air, which was supplied from a 2-inch manifold under 10 pounds of pressure per square inch, and regulated by means of a needle valve. In order to minimize evaporation losses, a gas-washing bottle was used to add moisture to the air before it entered the fermenter.

RESULTS

A comparison was made between the results obtained with *T. utilis*, using the new fermenter described in this paper and a previously used fermenter. The latter has been described (Stubbs, Noble, and Lewis, 1944) and consists essentially of an upright copper tube 36 inches tall by 6 inches in diameter with a working capacity of 8 to 10 liters. A 4-inch porous disc ("alfrax," coarse grade) is fitted in a false bottom at the base of the tube and serves to disperse the air for aeration. A water jacket provides control of temperature. The results (table 1) indicate the marked superiority of the new fermenter employing mechanical agitation for air dispersion with respect to a continued higher propagation rate, and particularly to the attainment of a much higher final yeast volume.

TABLE 1

Comparison of new fermenter (air dispersed by mechanical agitation) with old fermenter (air dispersed by porous disc) for propagation of Torulopsis utilis

TIME	NEW FERMENTER				OLD FERMENTER			
	Total vol. of culture	Yeast volume	Total yeast (dry wt)	Propagation rate (hourly yeast increase)	Total vol. of culture	Yeast volume	Total yeast (dry wt)	Propagation rate (hourly yeast increase)
hr	*ml*	*ml*	*g*	%	*ml*	*ml*	*g*	%
0	750	0.30	3.0		3,750	0.30	15.0	
1	750	0.12	4.2	40	3,750	0.34	17.0	13
2	758	0.57	5.8	38	3,775	0.43	21.6	27
3	790	0.82	8.6	48	3,850	0.61	31.3	45
4	838	1.10	12.3	43	4,000	0.77	41.1	31
5	913	1.53	18.6	51	4,200	1.00	56.0	36
6	1,030	2.00	27.5	48	4,425	1.28	75.5	35
7	1,220	2.50	40.7	48	4,735	1.54	97.2	29
8	1,455	2.90	56.3	38	4,960	1.67	110.5	14
9	1,720	3.43	78.7	40	5,100	1.80	122.5	11
10	2,060	3.70	101.5	29	5,100	1.95	132.5	8

SUMMARIZED DATA	NEW FERMENTER	OLD FERMENTER
Total sugar supplied	190 g	228 g
Dry yeast produced	98.5 g	117.5 g
Yield of yeast (based on sugar supplied)	51.8 %	51.5 %
Increase of yeast over inoculum	32.8 ×	7.8 ×
Average propagation rate (hourly increase)	42.3 %	24.9 %

The progressive decrease in growth rate after the sixth hour in the fermenter previously used is a phenomenon that is associated with inadequate aeration and one that has been noted in numerous yeast-culturing experiments. The rate of aeration used in the old fermenter in terms of total yeast present was well in excess of that used in the new fermenter in order that the volume of air in the former might be sufficient. The observed differences in results between the old (porous disc type) fermenter and the new (mechanical agitation type) fermenter are therefore regarded as being primarily due to the manner of dispersion of air.

Saccharomyces cerevisiae was cultured in molasses and in asparagus juice media to demonstrate the possible usefulness of the new fermenter for use in research in connection with the propagation of bakers' yeast (table 2).

The asparagus juice appeared to be a better culture medium than the molasses, as shown by the fact that the average propagation rate was 31.0 per cent in the former as compared with only 24.0 per cent for the latter. A more striking difference is revealed by comparing the respective dry weights of yeast produced in 12 hours' time. The increase of dry yeast was 23.8 times the weight of the inoculum in the asparagus juice but only 12.1 times that in the molasses.

TABLE 2

Comparison of molasses with asparagus butt juice media for propagation of Saccharomyces cerevisiae in new fermenter

SUMMARIZED DATA	MOLASSES MEDIUM	ASPARAGUS JUICE MEDIUM
Total sugar supplied (12 hr)	68.8 g	150 g
Dry yeast produced	31.5 g	69.3 g
Yield of yeast (based on sugar supplied)	45.8 %	46.2 %
Increase of yeast over inoculum	12.1 ×	23.9 ×
Average propagation rate (hourly increase)	24.1 %	31.0 %

It should be pointed out that differences obtained in comparing different media, as noted above, or that may be obtained in evaluating various nutritional or environmental factors in propagating yeast are likely to be at least partially obscured when the conventional type of fermenter is used, wherein aeration (air dispersion) is a limiting factor.

DISCUSSION

Yeast volumes of *T. utilis* of about 3.6 ml per 15 ml of culture liquid were obtained in the new fermenter with the propagation rate maintained at 40 per cent or more per hour. An average of approximately 4 billion cells per ml of culture liquid were found by direct microscopic count to correspond to the yeast volume of 3.6 ml. This appears to be twice the cell concentration previously reported as a maximum by Thaysen (1945), who has shown that a cell concentration of about 2 billion cells per ml represents the upper limit for maintenance of the normal growth rate, using ceramic blocks for air dispersion.

A yeast volume as high as 4.75 ml was reached in one experiment which was started with a relatively large inoculum, but the usual propagation rate could not be maintained at levels much above 3.6. This result suggest the possibility of still further improvement in air dispersion. Other factors, however, may influence the propagation rate adversely at such extremely high concentrations of yeast cells.

The effects of violent agitation caused by the rapid mechanical stirring in the new fermenter, as compared with the effects of mild agitation accompanying ordinary aeration, cannot be evaluated separately from the influence of improved air dispersion on yeast growth. The rates of yeast propagation observed in the new fermenter are probably not significantly greater than comparable rates obtained with equipment employing ordinary aeration at low cell concentrations where such aeration is adequate. Therefore the finer and more uniform air dispersion effected by mechanical agitation rather than mechanical agitation itself is regarded as primarily responsible for the excellent results obtained.

Use of the new fermenter should permit nutritional and related investigations to be conducted with greater confidence than heretofore, because aeration is more nearly optimum. The apparatus will probably also be found useful for the investigation of various microbiological fermentations, such as the submerged culture production of antibiotics by certain bacteria and molds.

SUMMARY

An improved laboratory fermenter for the culturing of yeast, employing mechanical agitation for the dispersion of air, with an operating capacity of 500 to 2,000 ml, has been described.

The results obtained in propagating *Torulopsis utilis* were markedly superior to those obtained with a previously described fermenter employing a porous disc for air dispersion.

Cell concentrations of *T. utilis* approximately twice as high as those previously reported were obtained in the new fermenter without diminishing the average propagation rate.

The more nearly optimum culturing conditions provided by the new fermenter, which makes possible the attainment of high cell concentrations and the maintenance of the normal propagation rate for prolonged periods of culturing, is ascribed to improved dispersion of air.

The new fermenter is recommended for use in yeast propagation investigations, and may be found suitable in such fermentations as the submerged culture production of antibiotics by bacteria and molds.

ACKNOWLEDGMENTS

The authors are indebted to Dr. E. C. Saudek of Consumers Yeast Company, Oakland, California, for his generosity in supplying molasses wort and bakers' yeast inoculum, and to Miss D. J. Hirschmann of this laboratory for direct microscopic cell counts of *T. utilis.* Special recognition is accorded to Mr. John Hoffman, Mr. Ernest Aeschlimann, and Mr. Paul Marsh for valuable assistance in the mechanical development and construction of the fermenter.

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THE ISOLATION OF ESCHERICHIA COLI PHAGE BY MEANS OF CATIONIC DETERGENTS[1]

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The action of synthetic detergents upon several strains of bacteriophage has been mentioned in a previous communication (Klein *et al.*, 1945). With the exception of gamma coli phage, the bacteriophages tested were highly susceptible to the germicidal activity of the various test compounds. The gamma phage, on the other hand, proved to be resistant to the highest concentrations of the detergents tested.

Because of this marked resistance of the gamma coli phage, various cationic detergents were used in attempts to isolate coli phage directly from sewage. Primarily, we were interested in ascertaining whether or not a coli phage may be isolated without the use of filtration.

MATERIALS AND METHODS

Raw sewage obtained from the sewage disposal plant constituted our source of phage. Only cationic detergents were employed as it has been shown that the anionic detergents are active against gram-positive organisms and have no activity against the tested gram-negative bacteria (Baker *et al.*, 1941*b*). The detergents[2] employed were zephiran, phemerol, cetyl pyridinium chloride (cepryn) and emulsol-607. The structural formulae for these compounds have been previously given (Baker *et al.*, 1941*a*, 1941*b*; Klein and Stevens, 1945). The detergents were used in a final concentration of 1:5,000. Zephiran and phemerol were supplied as a 1:1,000 dilution, whereas the other compounds were diluted to 1:1,000 in saline and stored as stock solutions. When used, 2 ml of detergent (1:1,000) were added to 8 ml of test material.

Initially, the liquid, raw sewage was passed through several thicknesses of gauze, centrifugated, and the supernatant separated into two samples.

(a) One sample was further divided into 5 parts, one being filtered (Seitz filters were used throughout), the others being used to make up detergent dilutions as described above. All samples were allowed to stand at room temperature for one-half hour and were then tested for sterility. When not in use, the samples were stored at 4 C. To determine the presence of phage, 0.1 ml was added to 10 ml of a 3-hour culture of *Escherichia coli* and incubated at 37 C for 3 hours. The lysis of the culture or the production of plaques indicated the presence of phage.

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[2] We wish to thank the following companies for their generous supply of the detergents: zepheran, Winthrop Chemical Co.; phemerol, Parke, Davis & Co.; cetyl pyridinium chloride, Wm. S. Merrell Co.; and emulsol-607, Emulsol Corp.

For the production of plaques, a 0.2-ml sample was removed from the phage-culture mixture after allowing a few minutes for adsorption of the phage to the organism. This sample was spread over the surface of 1.5 per cent extract agar by means of a glass spreader. Tenfold serial dilutions were used to determine the titer of the phages. After incubation at 37 C for 24 hours, each sample was retreated, i.e., the filtered sample filtered, and the other samples used to make up 1:5,000 dilutions with their respective detergents. This procedure was repeated at least three times.

(b) The second sample (50 ml) was added to an equal volume of extract broth and to this mixture 2 ml of a 3-hour culture of *E. coli* were added. This preparation was then incubated at 37 C for 24 hours, centrifugated, and divided into 5 samples. These samples were then treated as described in (a) above.

TABLE 1

The isolation of coli phage from sewage

DETERGENT DILUTION (FINAL)	WITHOUT ANY PRIMARY INCUBATION IN THE PRESENCE OF E. COLI			WITH A PRIMARY INCUBATION IN THE PRESENCE OF E. COLI		
	Passage			Passage		
	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3
Filtered (Seitz filters)	–*	–	–	+	++	++
Zephiran 1:5,000	+†	++	++++	+	++++	++++
Phemerol 1:5,000	–	–	–	+	+	+
Cepyrn 1:5,000	+	++	++++	+	++++	++++
Emulsol 1:5,000	+	++	++++	+	++++	++++

* –, no phage present as indicated by the lack of lysis or the formation of plaques.

† +, phage present, lysis, and plaques up to dilution 10^{-2}.

++, phage present, lysis, and plaques up to dilution 10^{-4}.

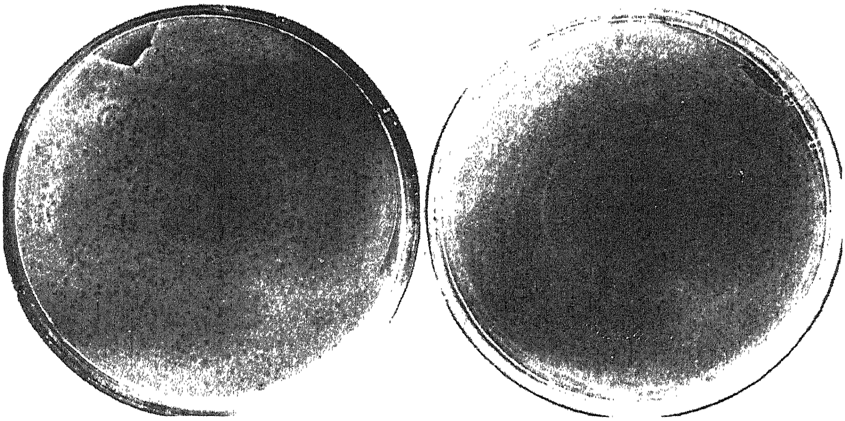
+++, phage present, lysis, and plaques up to dilution 10^{-6}.

++++, phage present, lysis, and plaques up to dilution 10^{-8}.

RESULTS

The results obtained by using cationic detergents for the isolation of coli phage are shown in table 1. This table is a summary of repeated experiments using the two methods described above. It is evident that several of these compounds may be used for the isolation of coli phage from sewage. However, the primary incubation of the sewage in the presence of a suceptible organism yields a greater amount of phage. By this method, phage in varying amounts was obtained from all preparations. Phemerol gave the smallest yield, filtration gave a slightly better yield, whereas the zephiran, cepryn, and emulsol-607 preparations, when used undiluted, caused the complete lysis of the culture and the formation of uncountable numbers of plaques. These compounds also yielded the best results when used for phage isolation without the preliminary incubation period. This second method, however, did not yield any phage by filtration or by the use of phemerol. The isolated coli phages do not appear to be pure-line phages as they caused the lysis of 3 out of 5 strains of *E. coli* when tested for lytic activity.

The phages resulting from the different methods of isolation were of interest as two different types of coli phage were isolated. From the primary incubation with *E. coli,* plaques were obtained which were mainly of large size with a few small-sized plaques present (figure 1, left). Without the primary incubation, the emulsol preparation gave similar plaques, but the zephiran and cepryn preparations gave plaques of small size only (figure 1, right).



(Photographs by Miss Stella Zimmer, College of Medicine, Syracuse University)
FIG. 1. LEFT: Large-sized Plaques RIGHT: Small-sized Plaques

TABLE 2

The activity of detergents on the isolated coli phage strains

DETERGENT DILUTION	PHAGE DILUTION 10^{-2}		PHAGE DILUTION 10^{-4}	
	Strain 103B	Strain 103E	Strain 103B	Strain 103E
Zephiran 1:5,000	40×10^7*	14×10^7	20×10^7	12×10^7
Phemerol 1:5,000	35×10^7	37×10^7	15×10^7	36×10^7
Cepryn 1:5,000	24×10^7	37×10^7	15×10^7	2×10^6
Emulsol 1:5,000	23×10^7	50×10^8	10×10^7	33×10^8
Control	30×10^7	50×10^8	15×10^7	30×10^8

* The number of phage particles per ml as determined by plaque counts.

It was thought that this difference might be due to a selective action on the part of the detergents. Accordingly the two phages were titrated against the various detergents. Final dilutions of a 1:5,000 detergent were tested against two phage dilutions, 10^{-2} and 10^{-4}, by allowing the phages to stand in contact with the detergent for 10 minutes. This mixture was then titrated and plated at a point beyond the bacteriostatic range of the detergent. The results obtained are summarized in table 2.

Of the two strains tested, strain 103 B (small-sized plaques) was not affected by any of the detergents at either 10^{-2} or 10^{-4} dilution. On the other hand, phage strain 103 E (large-size plaques) showed a different inactivation pattern. The detergents zephiran, phemerol, and cepryn caused at least a tenfold drop in phage titer with both of the test dilutions of phage. Emulsol appeared to have little effect on either of the test dilutions.

DISCUSSION

The results show that cationic detergents may be used for the isolation of coli phage from sewage. These results also suggest that the detergents may be preferred for the isolation of this phage because of the greater quantity of phage obtained and the elimination of filtration.

The failure to isolate phage by filtration in method (a) is understandable as filtration is known to adsorb phages. This was evident since phage was obtained when the sewage was allowed to incubate in the presence of the susceptible organism as in method (b). The inability to procure phage with the detergent phemerol by method (a) is as yet inexplicable as the results show that this detergent does not cause any greater inactivation than the other test compounds.

Because of the size of the plaques produced, we are assuming, until further studies prove otherwise, that the two strains of coli phage isolated are different and are possibly alpha and gamma coli phages. If this proves to be correct, the phage showing the smaller plaques would be the gamma phage, whereas the large plaques would be due to alpha phage (Delbrück, 1945). Further, the gamma phage was previously shown to be resistant to the detergents (Klein *et al.*, 1945), and this is substantiated by our present findings. The alpha phage is possibly less resistant to the detergents. It will be of interest to ascertain if a known alpha phage shows a different inactivation pattern than the gamma phage. Studies on this question are now in progress.

From the data obtained, it would appear that the detergents emulsol, zephiran, and cepryn are favored for the isolation of the coli phage. Phemerol is not recommended because of the uncertain results obtained.

CONCLUSIONS

Coli phage may be isolated from sewage with or without primary incubation in the presence of its susceptible organism by the use of cationic detergents.

The detergents emulsol-607, zephiran, and cetyl pyridinium chloride (final dilution 1:5,000) are recommended for the isolation of coli phage from sewage.

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A DILUTION PLATE COUNTING METHOD FOR CERTAIN STRAINS OF BACTERIUM TULARENSE[1]

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Turbidimetry and titration of virulence for animals have constituted the only methods available for assaying cultures or suspensions of *Bacterium tularense.* Although these methods are generally recognized to be inadequate for the measurement of viable organisms, no dilution plate counting method has been developed for this organism. Larson (1945) has shown that the end points provided by growth from serial dilutions on glucose cystine blood agar (Francis, 1928) approximate those of mouse titrations, but this medium was not recommended for plate counts, nor have we obtained consistent results with it. Downs, Coriell, and Chapman (1946) have used successfully a surface plate counting technique employing a modification of Francis' medium in which cystine is replaced by cysteine. As a consequence of our finding that reducing agents markedly lower the minimum effective inoculum of certain strains (Snyder, Penfield, Engley, and Creasy, 1946), we have also been able to devise a plating medium which offers two advantages: it is easily prepared from common dehydrated constituents, and its transparency facilitates counting of colonies with the aid of the usual colony counters.

Broth is prepared with 2 per cent Difco bacto peptone, 1 per cent sodium chloride, 0.1 per cent glucose, and 0.1 per cent cysteine hydrochloride, and adjusted to pH 6.8 to 7.0 with sodium hydroxide. Two per cent bacto agar is added and the medium sterilized and the agar melted by autoclaving at 121 C for 15 minutes. It is dispensed into sterile 100-mm petri plates in thick layers (about 30 ml per plate). After solidification, the plates are opened, inverted, and dried for about 1 hour in a clean incubator at between 50 and 60 C.

At least three plates are inoculated each with 0.1 ml of appropriate serial tenfold dilutions of the test material in a diluting fluid consisting of 1 per cent sodium chloride and 1 per cent gelatin. The fluid is spread over the surface of the agar with a sterile, cane-shaped, glass spreader, which obviates the need for opening the plates more than slightly. Inoculated plates are allowed to stand for about 30 minutes; then they are inverted and incubated at 37 C. Colonies are counted after 3 days, at which time they are 1 mm or more in diameter.

The plates should be inoculated on the day of preparation, before excessive oxidation of the cysteine has occurred and before a few unavoidable contaminants have developed. Cysteine hydrochloride may be replaced by 0.01 per cent thioglycolic acid, but in this case 4 days of incubation are required. The convenional type of poured plate was not found satisfactory because of the extremely low development of subsurface colonies.

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[1] Studies conducted at Camp Detrick, Frederick, Maryland, between February and December, 1945.

[2] 1st Lt., SnC; 2nd Lt., SnC; T/4, WAC; and PhM3/c, USNR; respectively.

The pleomorphism of *Bacterium tularense* and the presence of very small units make evaluation of the method by means of the direct microscopic count impracticable. We have used virulence titrations for this purpose, assuming an equivalence of infective and reproductive units. Forty-three comparisons were made with the highly virulent strain Schu, using 3 to 5 plates, and inoculating 6 mice ntraperitoneally, with each appropriate dilution. The number of organisms per nl of original suspension was calculated from the plates in the usual manner, and rom the animal titrations by the method of Stevens (Fisher and Yates, 1943). The ratios of organisms determined by plate count to organisms determined by animal titration were analyzed statistically, and gave a geometric mean ratio of 0.85 with a standard error of the logarithms of the ratios of ±8.0648–10. This mean ratio does not differ significantly from unity; hence the results provided by the two methods may be considered identical for practical purposes. Since clumps of organisms could not be observed microscopically, it is improbable that these two methods of enumeration would agree so closely unless both were dependent upon the individual organism as a unit. The standard error of the plate count, using 4 plates for each dilution, has averaged ±9 per cent of the mean at a mean count of 150 colonies per plate.

This counting method was tested briefly with 27 other strains, but the results were not subjected to statistical analysis. Ten highly virulent strains and three strains of lowered virulence appeared very similar to strain Schu in the rate of development of colonies and in the magnitude of counts obtained with suspensions of equivalent turbidity. On the other hand, three highly virulent strains and eleven of lowered virulence either gave irregular and markedly lower counts, with considerable delay in the development of colonies, or else failed entirely to grow except with excessive inocula. The addition of blood failed to improve the results with these strains.

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THE EFFECT OF SERUM UPON DISSOCIATION IN BRUCELLA ABORTUS: A DEMONSTRATION OF THE ROLE OF SELECTIVE ENVIRONMENTS IN BACTERIAL VARIATION[1]

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A previous report (Braun, 1946) on studies with *Brucella abortus*, strain 19, led to an interpretation of dissociation in terms of spontaneous appearance of variants and their subsequent establishment under the control of inherent and environmental factors governing population dynamics. One outgrowth of these studies was an attempt to produce *Brucella abortus* vaccine from smooth clones with low dissociation index (D.I. = the percentage of dissociated types within a population after 10 days of growth in broth; for details see Braun, 1946). In the course of these additional studies it was observed that vaccines made from some clones were superior in certain characteristics to control vaccines made from Bureau of Animal Industry strains; but it was also found that the intense growth period on Blake bottles, necessary in the manufacture of vaccines, can produce a change in the selected inherent characteristics of the material used by permitting the establishment of smooth organisms with changed dissociation indices. Therefore, it became desirable to search for means to prevent changes in growing populations, i.e., an environment had to be found which would permit the propagation of the selected type only and would prevent the establishment of changed types (mutants). It was thought that such selective environments might be produced by adding to cultures material containing antibodies for types which are not desired.

Previous *in vitro* studies by Dawson and Sia (1931) and Alloway (1932) have demonstrated that R types of pneumococcus can be "transformed" to S types if grown in the presence of anti-R serum, or in the presence of normal swine serum, which according to Alloway contains R antibodies. (Avery, MacLeod, and McCarty, 1944, later obtained results which suggested that factors other than R antibodies are involved.) The following experiments were, therefore, designed to investigate the effect of certain antisera and normal sera upon the dissociation of *Brucella abortus*.

EXPERIMENTAL[2]

For the production of antisera, rabbits were inoculated with suspensions of either smooth, rough, brown, or a mixture of rough and brown, all of which had originated from the progeny of a transplant of strain 19-12A of *Brucella abortus*,

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[1] This work has been supported in part by the Bureau of Animal Industry, USDA, under co-operative agreement with the Regents of the University of California.

[2] For general procedures see Braun, 1946.

which had been received from the Bureau of Animal Industry on October 19, 1943. Inoculations were made weekly over a period of 3 weeks. One week after the last inoculation the rabbits were bled from the heart.[3] The antisera were then added in varying amounts to broth cultures, which were inoculated with

TABLE 1

Summarized tabulation of the effects of serum or plasma upon dissociation

STRAIN USED	TYPE OF SERUM OR PLASMA ADDED TO BROTH CULTURES	AMOUNT ADDED TO 5 ML OF BROTH	AVERAGE D.I. PER CENT	NUMBER OF CULTURES
S, Clone 2583	none	none	20	48
	Normal serum from *rabbit*	0.05 to 1.0 ml	0†	60‡
	R antiserum from *rabbit*			
	Br antiserum from *rabbit*			
	R + Br antiserum from *rabbit*			
	S antiserum from *rabbit*	0.05 to 1.0 ml	2	8
	Normal serum from *cow*	0.1 to 1.0 ml	0	58
	Normal plasma from *cow*	0.1 to 1.0 ml	0	16
	Normal serum from *hog*	0.1 to 1.0 ml	0	6
	Normal serum from *goat*	0.5 ml	0	4
	none*	none	61	2
	Normal serum from *cow**	0.2 to 0.5 ml	0	7§
S, Clone 2907	none	none	20	6
	R antiserum from *rabbit*	0.05 to 1.0 ml	0¶	21‖
	Br antiserum from *rabbit*			
	R + Br antiserum from *rabbit*			
	S antiserum from *rabbit*	0.05 to 1.0 ml	4	8
S, 19–17 A (B.A.I. culture)	none	none	29	6
	R antiserum from *rabbit*	0.05 to 1.0 ml	0	20
	Br antiserum from *rabbit*			
	R + Br antiserum from *rabbit*			
	S antiserum from *rabbit*	0.05 to 1.0 ml	<1	8

Total number of cultures with serum (or plasma): 221.

Number of cultures showing dissociation in the presence of more than 2 per cent serum or plasma (except S serum): 4.

* Kept at 38 C.

† D.I. of 2 cultures with 0.05 ml Br antiserum added: less than 1 per cent.

‡ D.I. of 1 *additional* culture with 0.5 ml Br antiserum: 12 per cent. D.I. of 1 *additional* culture with 0.5 ml R + Br antiserum: 4 per cent. D.I. of 1 *additional* culture with 0.1 ml R + Br antiserum: 3 per cent.

§ D.I. of 1 *additional* culture with 0.5 ml serum added: 7 per cent.

¶ D.I. of 2 cultures with 0.5 ml R + Br antiserum added: less than 1 per cent.

‖ D.I. of 1 *additional* culture with 0.05 ml R antiserum: 12 per cent.

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[3] In agglutination tests rough and brown antigens exhibited a definite reaction with all antisera used. This reaction differed somewhat from the typical agglutination observed when S antigen and positive S antiserum were used, i.e., the agglutination was less pronounced, but nevertheless significantly different from the settling of control antigens without antiserum.

equal amounts of a suspension of clone 2583, a clone with a fairly high dissociation index under standard conditions. Table 1 summarizes the results of a series of such experiments. Except for a very small number of cultures (1 in 60), all cultures to which antiserum of rough or brown, or even normal serum, had been added showed absence of dissociation, but control cultures without serum showed considerable dissociation. The addition of serum, in concentrations as low as 2 per cent, thus usually suppresses the establishment of dissociated types. It appeared unnecessary to produce specific antisera against rough or brown, because the initial results indicated that normal serum contains factors which suppress rough and brown types. This was further confirmed in extensive tests (summarized in table 1), which revealed that normal serum or plasma from cows, as well as from hogs and goats, suppress the establishment of rough and brown types efficiently.[4] (The presence in normal serum of factors reacting with rough and brown types is also supported by the reaction observed in agglutination tests.)[5] The addition of 0.01 ml or less of serum or plasma to 5 ml of broth did not prevent dissociation.

In the case of the addition of S antiserum (table 1) some dissociated types do establish themselves; presumably the suppression of S types due to the presence of S agglutinins is stronger than the suppression of rough and brown types by factors normally present in serum.

In the rare instances in which dissociated types occur in the presence of more than 2 per cent serum, the variants usually differ from the types commonly observed. A "whitish brown" type (mucoid), a "transparent" type (mucoid), and a "brownish rough" type have been isolated from such exceptional cultures. Presumably these uncommon variants cannot establish themselves when they are in competition with arising rough and brown types. Only when the establishment of the latter types is prevented through the factors present in serum do the uncommon types attain a positive selection value which permits their establishment.

Recently, Huddleson *et al.* (1945) published a detailed report dealing with the bactericidal action of bovine serum and plasma on *Brucella abortus*. Although the concentration of serum in the experiments reported in the present paper was very much smaller than the concentrations used by Huddleson *et al.* for the demonstration of bactericidal activity, it appeared necessary to test whether our results might be due to the bactericidal action of serum or plasma. Such tests appeared to be of special significance since it had been previously shown that factors which affect growth-rates also affect dissociation (Braun, 1946). The absence of dissociation in cultures to which serum has been added could, therefore, be due merely to a general retardation of growth. Experiments designed to test the possible relationship between the general bactericidal activity of serum and the suppression of dissociation, however, revealed that the suppression of dissociation in cultures with serum is not due to any general bactericidal or bacteriostatic acitivity. First, it was found that the growth rates of smooth types were not affected by the addition of serum sufficient for the suppression of dissociated types (table 2). Second, Huddleson *et al.* had demonstrated that the bactericidal activity depends on the presence of complement and that the removal of complement by heating at 56 C for 1 hour, or by filtration through a Berkefeld W filter, removed the bactericidal action. Results compiled in table 3 show that neither heating nor filtration removed the factors responsible for the suppression of dissociated types in our experiments.

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[4] Horse serum and chicken serum do not suppress dissociation so effectively as sera from other animals tested. Uncommon types of variants were found in many cultures to which chicken serum had been added.

[5] See footnote 3.

TABLE 2

Growth in the presence and absence of serum

	AT START OF CULTURE	AFTER 3 DAYS	AFTER 10 DAYS	
Total number of cells*	231 M†	460 M	1.450 B‡	Without serum
Total number of cells*	231 M	488 M	1.413 B	With 0.2 ml normal rabbit serum
Total number of cells*	231 M	478 M	1.500 B	With 0.2 ml normal cow serum

* All figures are averages of 4 cultures.
† Millions.
‡ Billions.

TABLE 3

The effects of filtered or heated serum upon dissociation

STRAIN USED	TYPE OF SERUM ADDED TO BROTH CULTURES	AMOUNT ADDED TO 5 ML OF BROTH	AVERAGE D.I. PER CENT	NUMBER OF CULTURES
S, clone 2583	none	none	20	48
S, clone 2583	Normal serum from cow, *filtered* through Berkefeld W filter	0.1 ml, *or* 0.2 ml, *or* 0.5 ml	0	12
S, clone 2583, 2907, 1863, & strain 19–17A	Normal and antisera from rabbit, *heated* 1 hour at 56 C	0,1 ml, *or* 0.2 ml, *or* 0.5 ml	0	50
S, clone 2583	Normal serum from cow, *heated* for 30 min at 62 to 63 C	0.5 ml	0	8

It could be assumed that the factors present in serum and plasma either prevent the change from smooth to rough or brown, or inhibit the establishment of the latter types, or that they do both. Whereas it has not been possible yet to investigate the first-mentioned possibility, it was proved by a series of tests that the establishment of rough and brown types is actually inhibited in the presence of serum. Cultures with and without serum were started with known percentages of smooth and rough organisms. Ten days later samples from these cultures were streaked on "2–1 agar" plates, and the percentages of smooth and rough colonies was determined. No increase in the percentage of roughs was found in cultures containing serum, whereas control cultures without serum showed a considerable increase of the percentage of roughs over smooth (table 4).

So far all attempts to suppress the growth of dissociated types on solid media, through the addition of serum to "2–1 agar," have failed. It has also been observed that the addition of 10 per cent of normal serum from nonvaccinated, noninfected cows to vaccines not only prevented the occurrence of dissociation but also caused a significant increase in viability during storage.

TABLE 4

The inhibitory effect of normal cow serum upon the establishment of R types

AMOUNT OF NORMAL COW SERUM ADDED PER BROTH CULTURES	PERCENTAGE OF ROUGH AMONG SMOOTH	
	At start of broth cultures	After 10 days
None	1	22
0.2 ml	1	<1
None	5	24
0.2 ml	5	3

DISCUSSION

The observed suppression of rough and brown variants in the presence of serum or plasma demonstrates the selective role of environmental conditions in bacterial variation. Under standard *in vitro* conditions, i.e., buffered broth,[6] rough and brown variants can establish themselves within an originally smooth population because of their greater viability (Braun, 1946). Under standard *in vitro* conditions rough and brown variants, therefore, have a higher selection value than smooth. The addition of serum or plasma obviously alters this selection value. Conditions similar to those observed when serum is added to broth appear to exist *in vivo*, where it has been observed that the S type only can be isolated after inoculation of a mixture of R and S variants (Henry, 1933). The environment thus has a profound effect on the "pattern" of bacterial variation by determining the selection value of variants that arise, regardless of the inherent tendencies for dissociation.

In this connection the problem of apparently successive orderly changes ("cycles") in bacterial variation may be discussed, a problem which has contributed much to what can now be considered previous misinterpretations of basic aspects of dissociation. The information now available makes it clear that of all spontaneously arising variants only those can establish themselves which have a higher selection value (growth rate or viability) than the original members of the population. For example, of all mutants which can occur within a smooth population rough and brown types usually have the highest selection value under standard *in vitro* conditions. According to some unpublished experimental observations on variants which arose from one clone, smooth types have the fastest growth rate, rough types a slower growth rate, and brown types a still slower growth rate; brown types have the highest viability (i.e., ratio of total number of

[6] Beef extract broth.

cells to viable number of cells per ml of broth), rough types a slightly lower viability, and smooth types have the lowest viability. Since the number of viable cells per ml is limited (Braun, 1946), rough and brown mutants which arise in the smooth population will replace the smooth type; during prolonged growth rough types will become predominant first and they will eventually be replaced by the brown type, causing an apparently orderly change from S → R → Br. Thus, competition between spontaneously arising mutants with different selection values, leading to the establishment of one predominant type (i.e., the one which shows highest viability under any given environmental conditions), will produce the appearance of successive orderly changes. Furthermore, since the rough type has only been able to establish itself within an originally smooth population through its higher viability, it now becomes obvious why it is usually so difficult to observe dissociation from rough to smooth *in vitro*. Should one smooth mutant arise in a rough population (as it probably does), it will have little chance to establish itself because it is competing with members of a population which have already a higher viability than the smooth mutant. If, however, environmental conditions are changed so that the smooth type attains a higher selection value than the rough type, as is the case in the presence of serum, then a change from rough to smooth can occur. In addition, certain smooth mutants can establish themselves in a rough population under standard *in vitro* conditions, namely, smooth mutants which have a higher selection value than members of the particular rough population in which they arise. Such smooth mutants have been observed (Braun, 1946).

The ability of spontaneously arising variants with positive selection value to establish themselves rapidly, within a population with lesser selection value under given environmental conditions, can be held responsible for the apparently specific "adaptation" which can be observed among bacteria under natural conditions, and can account for the occurrence of considerable variation when bacteria are removed from their normal environment and are grown under laboratory conditions. Removal from their normal environment apparently alters the selection value of arising variants; members of the original population cease to be the ones with greatest viability or growth rate, and arising mutants which formerly were unable to compete with the "normal population" can now establish themselves. The so-called "normal type" is thus merely one variant which has been able to establish itself under natural conditions in response to the forces of selection.

SUMMARY

Serum or plasma of normal cows, rabbits, hogs, and goats was found to contain factors which suppress the establishment of rough and brown variants of *Brucella abortus*. When small amounts of serum or plasma were added to smooth broth cultures, which normally would show considerable dissociation after 10 days, no dissociation occurred.

The suppression of dissociated types by serum or plasma is not due to factors which are responsible for the bactericidal action of sera or plasma.

These results form the basis for a discussion of the selective role of environments in bacterial variation, the appearance of *apparently* successive orderly changes during dissociation, the difficulties encountered in reversing the direction of dissociative changes, and the apparently specific adaptation of bacteria under natural conditions.

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THE REDUCTASE METHOD FOR THE DETERMINATION OF PENICILLIN CONCENTRATIONS IN BODY FLUIDS

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Methods for determining concentration of penicillin in blood serum or other body fluids are based on one of two, or a combination of the two, effects of the antibiotic on a susceptible organism. A well-known and widely used method is that of Rammelkamp (1942). Among the various modifications of this type of test are those described by Rake and Jones (1943) and Kirby and Rantz (1944). These tests make use of the inhibition of the hemolytic activity of susceptible strains of streptococci by penicillin in body fluids. Another commonly used method employs the inhibition of growth principle as applied to a susceptible organism. Such methods have been reported by Joslyn (1944), by Lee, Foley, Epstein, and Wallace (1944), and by Randall, Price, and Welch (1945). These methods may make use of special devices to estimate turbidity or may simply report the presence or absence of growth of the test organism.

Each of these types of methods has merit, and each has certain disadvantages. In general, a satisfactory test will embody the use of some means to determine the effect of the antibiotic on the test organism that can be reproduced with a minimum of confusion by workers using the test. The increasing frequency of requests for penicillin blood or urine levels has made it necessary for many technicians, some of whom are not too familiar with antibiotic assay methods, to make occasional determinations. Many of these workers do not have turbidimeters and are not certain as to where the end point of growth occurs in tests in which growth or turbidity is the determining factor. Still this type of determination seems better suited to the laboratory doing only occasional tests, especially if *Bacillus subtilis* is used as the test organism, as recommended by Randall, Price, and Welch, than one based upon hemolysis by a beta streptococcus. Disadvantages of the Rammelkamp method, or modifications of it, are the need for a supply of fresh blood cells and the necessity for having a culture of hemolytic streptococcus in a state favorable for hemolysis at all times. *Bacillus subtilis* does not have to be transferred daily, and care need not be taken to keep it in a condition for optimal hemolysis. No fresh blood cells are required, although the final reading when all factors are properly controlled is clear-cut and beyond criticism. When the presence or absence of growth is the determining factor in reading the end point, considerable confusion may result even though read by experienced workers. This is magnified by the fact that a heavy inoculum is used in seeding the tubes. A desirable attribute of a test of this sort is a short incubation period so that there will be a minimum of opportunity for the breakdown of the antibiotic at incubation temperature. Modifications of the Rammelkamp method embody this consideration.

With all of these things in mind we set out to find a test that would embrace the best points in the present tests and omit as many as possible of the drawbacks. We desired a test that could be read in a short time or could be left in the incubator overnight before a reading was made. This would overcome the possibility of destruction of penicillin, or other antibiotic, due to long exposure to incubation temperature; it would allow the laboratory to report on a penicillin level within a matter of 4 to 6 hours in critical cases. We wanted a test in which the end point would be so clear-cut that there would be no confusion among workers as to the interpretation. A test was desired that would measure some end point other than turbidity, and finally we wanted to make use of a test organism that would not require constant attention or even daily transfers in order to give accurate and reproducible results.

A consideration of many of the biochemical and physiological activities of suitable test organisms led, after many others had been tried, to the selection of the reductase activity of the suceptible bacteria. This test is based upon the familiar methylene blue, or reductase, test for estimating the bacteriological quality of fluid milk. In this test the number of bacteria in milk may be estimated by the length of time necessary for the reduction of a solution methylene blue, or more recently resazurin. Our method makes use of a massive inoculum of organisms in a sterile milk medium containing methylene blue and determination of the inhibition of the bacteria by lack of reduction of the dye. Nearly any susceptible bacterium may be used because reductase production is a common activity among bacteria. *Bacillus subtilis* was finally selected as the most suitable because there was no significant difference in the results obtained after 4 to 6 hours' incubation when an 18-hour culture was used or a week-old broth suspension consisting largely of spores of the organism.

As the test medium we first made use of sterile skim milk to which had been added methylene blue thiocyanate solution (Standard Methods of Milk Examination, A.P.H.A.). The results obtained with this very simple medium were encouraging and satisfactory; however, it was found that by the addition of a suitable peptone and adequate agar to reduce convection currents in the medium, results could be obtained in a shorter time and would remain constant over a longer incubation period, if it was desired to incubate overnight rather than for a few hours. The medium which we have called Reductase Medium is composed of

Milk powder	80 g
Trypticase, B.B.L. (tryptic digest of casein)	10 g
Agar	0.5 g
Methylene blue thiocyanate (National Aniline certified dye:1 tablet per 2.2 liter)	
Distilled water	1,000 ml

The technique of the test follows the scheme described by Randall, Price, and Welch (1945). The first tube contains 0.5 ml of the fluid to be tested and is not diluted with medium; tube 2 contains 0.5 ml of medium to which are added 0.5 ml of the material under test. From this tube serial twofold dilutions are made through at least 8 tubes using the foregoing medium as diluent. Similar dilutions are made in three tubes which are used with a penicillin-inactivating substance (Chandler, Price, and Randall, 1945). A standard is made for comparison by diluting a penicillin of known potency (working standard) to 1 unit per ml in buffer solution. This 1-unit standard is diluted in exactly the same manner as the fluid under examination. When the dilutions are made, 1.5 ml of the medium, which has been inoculated with 5.0 ml per 100 ml of a culture of *Bacillus subtilis*[1] (this culture should be at least 18 hours old and may be several days old), is added to each tube in the test. For the control series using the inhibitory substance, the medium is inoculated with the same strength of the *Bacillus subtilis* and 0.25 ml of a 4 per cent aqueous solution of clarase[2] are added before distributing it into the tubes. The tubes are incubated in a water bath or incubator at 37 C for 4 hours and observed. At this time there is usually sufficient differentiation to make an accurate reading possible. We have read again after 5 or 6 hours of incubation and finally after 18 hours' (overnight) incubation. The end point is the last tube which is still blue. This indicates that there has been no growth, or at least insufficient growth, of the test organism to produce the reductase enzyme. By repeated comparisons with the method of Randall, Price, and Welch we have found this test gives almost identical readings without the disadvantage of a question as to whether a slight amount of turbidity is due to growth or whether it may be inoculum or debris.[3] It should be remembered that appreciable amounts of hemoglobin will cause a reduction of the methylene blue, and for that reason serum must not be badly hemolyzed and should not contain large numbers of blood cells.

TABLE 1

Penicillin determination

	1	2	3	4	5	6	7	8	CLARASE		
									9	10	11
Standard units.	0.5	0.25	0.125	0.65	0.03	0.015	0.007	0.003	0.5	0.25	0.125
Standard.......	—	—	—	—	—	—	R	R	R	R	R
Serum 1........	—	—	—	—	—	R	R	R	R	R	R
Serum 2........	—	—	—	—	R	R	R	R	R	R	R
Urine 1:10......	—	—	—	—	R	R	R	R	R	R	R

—, not reduced (medium blue).
R, reduced (medium white).

The concentration of penicillin in an unknown is calculated as described in the paper by Randall, Price, and Welch. That is, the concentration of penicillin in the unknown is determined by comparison of the end point of the unknown with that of the standard. An example is given in table 1.

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[1] *Bacillus subtilis* strain from the Northern Regional Research Laboratory.

[2] Clarase is an enzyme preparation obtained from Takamine Laboratories, Inc., Clifton, New Jersey, for penicillin inactivation. Penicillinase may be used in place of clarase.

[3] For reasons we have been unable to explain, the end point obtained with this medium is occasionally one tube lower than that with the Randall, Price, and Welch medium to which we have made many comparisons. It is hoped that the use and study of this test by others will lead to its improvement to overcome this difficulty.

The example in the table shows that there was complete inhibition of reductase activity in the sixth tube. This represents 1 unit per ml. Serum 1 showed one-half of this activity (reduction in the sixth tube but not in the fifth) and therefore contains 0.5 of a unit per ml, and serum 2 inhibited in the fourth tube, but not in the fifth, and therefore by comparison with the standard contains 0.25 of a unit per ml. The inhibition by the urine specimen tested was likewise in the fourth tube, but this was using a 1:10 dilution, so the result must be multiplied by 10, or 2.25 units per ml. Other dilution schedules may be employed in order to determine the concentration in the unknown more closely.

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NOTE

THE USE OF THE RODENT-ADAPTED MEF1 STRAIN OF HUMAN POLIOMYELITIS IN NEUTRALIZATION TESTS WITH SERUM OF APPARENTLY NORMAL AFRICAN NATIVES

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Hudson and Lennette (Am. J. Hyg., **17**, 581) found that 18 of 19 sera from Liberian Negroes, in "qualitative" tests in monkeys, neutralized the M.V. simian-adapted virus, a fact which supported their conclusions, based on many series of tests on sera from regions where epidemics are infrequent, that the virus and poliomyelitis are widely distributed and generalized immunization continues at an equal pace (Am. J. Trop. Med., **18**, 35).

The MEF1 rodent-adapted strain of human poliomyelitis virus, isolated from a case in the Middle East Forces of the British Army, has been described by Schlesinger, Morgan, and Olitsky (Science, **98**, 452). Sera from convalescents and from normal white adults residing in the United States, Canada, and North Africa neutralized it as well as the rodent-adapted Lansing strain (Fed. Proc., **3**, 99). The present paper shows the results of neutralization tests in mice against MEF1 virus with sera collected from 72 apparently healthy natives of British West Africa, 1 to 40 years of age. Although cases of disease suspected as poliomyelitis occur there, the sera represent random sampling of a "healthy" community. Of the 72 sera, 8 were from natives 1 to 10 years of age; 24, 10 to 20 years; 21, 20 to 30 years; and 19 from Negroes aged 30 to 40 years. In addition, 3 sera were studied from patients convalescent from suspected poliomyelitis occurring locally.

The test was carried out as follows. Equal parts of undiluted serum and virus dilutions were mixed, the mixtures kept at 37 C for 2 hours, and 0.03 ml injected intracerebrally into 7 mice per dilution. A serum was designated as positive if it neutralized 100 lethal doses of virus.

Preliminary tests were made to confirm the identity of the poliomyelitis strains—tests that should be performed in view of the danger of contaminating test materials with spontaneous viruses found in mice in nature. Lansing- and MEF1-virus-immune (convalescent) monkey serum neutralized both Lansing and MEF1 viruses. Normal rhesus monkey serum was negative, in 21 separate tests, against 10 to 400 M.L.D. of virus. The sera deriving from normal native Africans failed to neutralize Theiler's virus, and the 3 obtained from poliomyelitis convalescents neutralized both the Lansing and the MEF1 strains. Finally, and characteristically, the LD_{50} of the Lansing and the MEF1 strains was at 1:1,000 but not 1:5,000 or higher dilution.

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All of the 72 sera from young and middle-aged, normal, African Negroes neutralized the Lansing type, rodent-adapted MEF1 strain of human poliomyelitis.

It would thus appear that the MEF1 strain could be substituted for the Lansing in mice in neutralization tests, as for example for epidemiological studies. The results with the Lansing strain are, in turn, in good agreement with those in monkeys with simian-adapted strains (Armstrong; Harvey Lectures, **36,** 39). Moreover, they support the finding that poliomyelitis virus is widespread—if the neutralizing antibody as tested for is regarded as a specific response to viral infection—even in the tropics where epidemics are not so commonly reported as in the temperate zone.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NEW JERSEY BRANCH

Princeton University, Princeton, New Jersey, April 18, 1946

The Relation of Hydrostatic Pressure to Specific Precipitation, Antibody Inactivation, and Protein Denaturation. *Frank H. Johnson*, Princeton University, Princeton, New Jersey.

Under a pressure of 10,000 lb per sq inch the rate of specific precipitation, in the presence of the synthetic hapten 1,3-dihydroxy-2,4,6,tris (azobenzene-4′-azobenzene-3″-arsonic acid) benzene, is greatly retarded at room temperature, although following the release of pressure it takes place in apparently the normal manner. The inactivation of anti-*Staphylococcus* hemolysin at 65 C is also retarded by this pressure. Lower pressures accelerate the rate of bacterial reproduction above the normal optimum temperature, presumably by counteracting a reversible denaturation of the limiting enzyme. Pressures up to 10,000 pounds retard the rate of disinfection of *Escherichia coli* at temperatures above 45 C, as well as at lower temperatures in the presence of quinine which accelerates the disinfection rate. The precipitation of purified human serum globulin at 65 C is accelerated by small concentrations of ethanol but is greatly retarded by pressures up to 10,000 pounds, with as well as without the alcohol. These results indicate large molecular volume changes of activation or of reaction, suggesting extensive changes in protein molecules in each case. The data were obtained in collaboration with Dr. Dan Campbell, Dr. George Wright, and Mr. Isaac Lewin.

The Agglutination of Certain Types of Intestinal Bacteria from a Healthy Human Being by the Individual's Own Serum. *Hazel B. Gillespie, M. Harriet Waugh, and Yvonne V. Serett*, Department of Bacteriology, New Jersey College for Women, Rutgers University, New Brunswick, New Jersey.

Aerobic and facultative cultures (85 rapid and 7 slow lactose-fermenting, coliform types; 13 gram-negative, non-lactose-fermenting rods; 9 easily grown, gram-positive rods) were isolated from feces from one healthy human being. Using agglutinating antigens, 571 tests were made with serum from the individual from whose intestinal flora the cultures had been secured, 40 with serum from two other human beings, 194 with rabbit serum, 160 with horse serum, 40 with serum from a new-born calf, and 26 with cow serum.

Sixty-six (72 per cent) of the 92 coliform and paracolon antigens gave titers ranging from 1:320 to 1:2,560 with "homologous" human serum, whereas none of the 22 non-coliform antigens gave titers higher than 1:80. "Heterologous" sera agglutinated many coliform antigens, but the titers obtained were lower.

These data suggest the possibility that certain strains of coliform bacteria which may inhabit the so-called normal intestine have greater invasive power or virulence than do some other similarly situated bacterial types.

Some Immunological Aspects of Antihistamine Substances. *R. L. Mayer, Philip C. Eisman, and Karen Aronson.* Research Laboratories, Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

Antihistaminic substances are able to prevent allergic manifestations in which histamine is considered to be the principal offender. It is generally supposed that they do not interfere with the antigen-antibody reaction but with histamine, which is liberated during this reaction.

In order to determine whether pyribenzamine interferes with bacterial immune processes, studies were made on its influence upon the therapeutic action of antipneumococcal serum in experimental pneumococcal infection, of sulfathiazole in pneumococcal and streptococcal infections, and of penicillin in streptococcal infections of mice; the production of immunity in mice after recovering from pneumococcal infections; the opsonic activity of leucocytes toward staphylococci; the capsular swelling reaction of Neufeld; the agglutination reaction between staphylococci and homologous staphylococcal rabbit serum; and the hemolytic activity of streptococci *in vitro*.

In no case was any influence upon these processes observed. These results show that therapy with pyribenzamine does not interfere with immunization activities of the body and the chemotherapeutic activity of antibacterial agents. Such studies might help clarify the question of relationship between sensitization and immunization.

EASTERN PENNSYLVANIA BRANCH

ONE HUNDRED AND EIGHTY-SIXTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, PHILADELPHIA, PA., MARCH 26, 1946

THE TUBERCLE BACILLUS AS AN INDICATOR ORGANISM IN QUANTITATIVE STUDIES OF AIRBORNE INFECTION. I. QUANTITATIVE AEROSOL SUSPENSION OF TUBERCLE BACILLI. *W. F. Wells*, Laboratories for the Study of Airborne Infection, University of Pennsylvania, Philadelphia, Pa.

An improved apparatus for the study of experimental airborne disease embodies the three essentials in an earlier model: first, an atomizer to suspend organisms in the air constantly; second, an inhalation chamber in which animals may be safely and conveniently exposed to this infection and samples collected; and third, an incinerating chimney to create constant airflow through the apparatus and to dispose of the organisms before the air is discharged into the room. Connecting lines are also adapted to the use of the apparatus as a testing device, using live animals for study of the effects of state and stage of airborne infection.

Auxiliary apparatus includes a settling chamber in which the sedimentation rate of the experimental nuclei can be determined. Settling velocity is given by the ratio of the volume count, determined by the air centrifuge, to the area count. Equivalent diameters of different-sized droplet nuclei produced in the aerosol flask can then be computed by Stokes law.

Tubercle bacilli are separated by culture in a revolving flask, containing glass beads. Filtered through a number 4 Whatman filter, the individual cells produced by this technique are counted by the Breed method.

II. QUANTITATIVE ENUMERATION OF TUBERCLE BACILLI IN VITRO. *Cretyl Crumb*, Laboratories for the Study of Airborne Infection, University of Pennsylvania.

Our problem involved the enumeration of singled tubercle bacilli of the Ravenel strain in pure culture. Media supplied by standard laboratories proved unsuitable for our purpose. Whether any medium could grow singled bacilli was first settled by inoculating progressive filtrates of standard cultures upon several media. Comparative tests of available media then disclosed a principle upon which an adequate formula was based. Sterile, fresh egg yolk is added with special aseptic precautions to an agar base, consisting essentially of the liquid medium used for the standard culture of these bacilli. This broth is a blend of equal parts of three Difco broths (brain heart infusion, tryptose phosphate broth, and nutrient broth) plus 5 per cent glycerol. For the solid medium (from which the glycerol is omitted) 1.5 per cent agar is added.

The suitability of the medium for our needs was tested by inoculation with aliquots of filtrates from 9 successive weekly generations of a standard culture. From counts of these suspensions of single cells, determined by the Breed method, and counts of colonies on this medium, we infer that, within the precision of measurement, there was no indication that any cell cultivated by the standard method would not grow. Thus the colonies represented quantitative counts.

III. QUANTITATIVE ENUMERATION OF TUBERCLE BACILLI IN VIVO. *H. L. Ratcliffe*, Department of Pathology, University of Pennsylvania.

Techniques and apparatus described by Wells and Crumb demonstrate that the respiratory system of the normal laboratory rabbit can serve as an additional means of determining the numbers of viable tubercle bacilli in droplet nuclei transported by experimental atmospheres. We have produced experimental infections resulting in the development of from 1 to more than 10,000 tubercles; but we have found that, for accurate enumeration, doses should range below 200 bacilli. Within this range tubercles reach diameters of 4 to 6 millimeters within 4 to 5 weeks and can be counted as readily as colonies of other organisms on an agar plate. Moreover, within this dosage range and time limit tubercles seem to develop as independent entities without significant evidence of fusion or hematogenous spread.

Under appropriate conditions of aerosol suspensions (Wells and Ratcliffe: 1945, Proc. Phil. Soc. Phila.) organisms are deposited quantitatively in alveoli; counts of tubercles corresponded to colonies obtained on the Crumb medium and to slide counts of suspensions. Thus we have reason to believe that any organism in our standard culture, observable under the microscope, will produce a visible tubercle in the lungs of normal rabbits if inhaled under the experimental conditions which have been described. All evidence thus far obtained supports the opinion that under the conditions of these experiments a single tubercle will develop from a single organism planted on alveolar walls.

DEVELOPMENT OF STREPTOMYCIN RESISTANCE OF SHIGELLAE. *Morton Klein and Leonard J. Kimmelman*, Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia 4, Pa.

ONE HUNDRED AND EIGHTY-SEVENTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, PHILADELPHIA, PA., APRIL 23, 1946

SEROLOGY OF RHEUMATOID ARTHRITIS. *A. D. Wallis*, Department of Orthopedic Surgery and Physical Medicine, School of Medicine, University of Pennsylvania, Philadelphia 4, Pa.

ALLERGY AGAINST INSULIN. *Mary H. Loveless*, New York Hospital and Department of Medicine, Cornell University Medical College, New York, N. Y.

ONE HUNDRED AND EIGHTY-EIGHTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, PHILADELPHIA, PA., MAY 14, 1946

STUDIES ON INHIBITION OF GROWTH BY STRUCTURAL ANALOGUES OF METABOLITES. *D. W. Woolley*, Rockefeller Institute for Medical Research, New York, N. Y.

BRUCELLA SUIS IN AERATED BROTH CULTURE

I. Preliminary Studies on Growth Assays, Inoculum, and Growth Characteristics in an Improved Medium[1]

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Camp Detrick, Frederick, Maryland

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The problem of growing *Brucella* in broth culture on a comparatively large laboratory scale for immunochemical studies is one which has received relatively little attention in the past. It was early decided that the factors of substrate, aeration, and inoculum were of particular significance. In addition, a continuous system of culture seemed feasible and desirable. But before such detailed investigations, it was necessary that the methods for assaying growth of the organism be evaluated and modified where necessary, and that an over-all picture of "normal" growth under optimum conditions of substrate and aeration be obtained for reference. It was for this purpose that the present study was undertaken.

METHODS

Although sparger aeration (i.e., bubbling of air through deep quantities of culture) was contemplated for subsequent studies, many preliminary investigations required a simple method to provide a high, yet constant and easily reproducible rate of air supply for the growing cultures. These conditions were best resolved by employment of shaker flasks. In this technique cultures were grown in 25 ml of medium contained in 250-ml Erlenmeyer flasks, which were aerated by continuous agitation on a reciprocating shaker apparatus at approximately 90 rpm (figure 1). Subsequent aeration studies proved that the aeration provided by shaker flasks was optimum for the medium employed and that the results so obtained could be used as an optimum standard for comparison with sparger aeration. All results in this report were obtained with shaker cultures.

The medium employed was a fortified modification (McCullough *et al.*, 1945) of tryptose broth (Difco): 2 per cent bacto tryptose, 0.5 per cent sodium chloride, 0.75 or 1.0 per cent glucose, 0.1 μg thiamine hydrochloride per ml, and distilled water. A single strain of *Brucella suis* was used throughout (received through the courtesy of Dr. I. F. Huddleson, his strain no. 1772-A). Incubation was at 37 C.

Plate counts were made by the usual poured plate method, using tryptose agar (Difco). Three plates were usually averaged per count. Plates were incubated 4 days.

Light transmittance was determined by means of the Coleman universal spectrophotometer, using matched 18-mm pyrex test tubes as cuvettes. Evalu-

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[1] Studies conducted at Camp Detrick, Frederick, Maryland, January to September, 1945.

[2] 1st Lt., Infantry, and 1st Lt., Army Air Force.

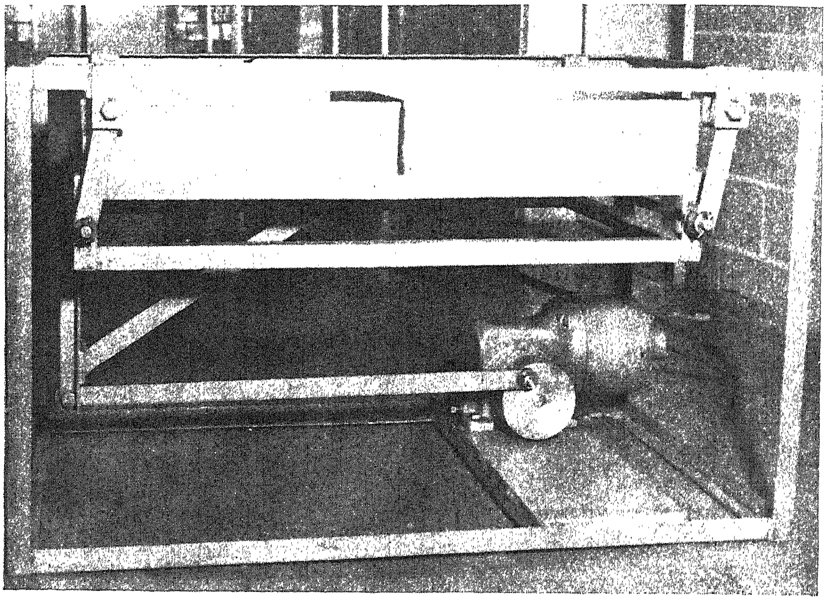

FIG. 1 TWO-TRAY RECIPROCATING SHAKER APPARATUS

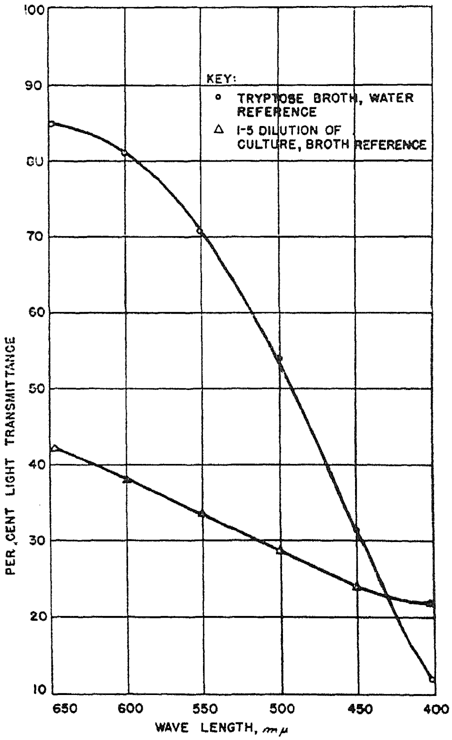

FIG. 2. SPECTRAL TRANSMITTANCE OF MEDIUM AND DILUTED CULTURE OF BRUCELLA SUIS

ations were made on both whole cultures and tenfold dilutions thereof, using whole and diluted medium, respectively, as references. Figure 2 shows spectral transmittance curves of two representative systems, which indicate that 650 mμ is the practical optimum wave length to be used. The effect of changes in pH between pH 6.9 and 9.0 on light transmittance was found to be negligible in both uninoculated medium and culture. The application of Beer's law to spectrophotometric evaluations of cultures was checked and found to be valid.

Catalase activity was determined by a modification of the method described by Huddleson (Huddleson and Stahl, 1943). After considerable study, the following method was found to give satisfactory results: To a 1.0-ml aliquot of 1:10 dilution of the culture in a 250-ml Erlenmeyer flask are added 15 ml of 3 per cent hydrogen peroxide. The flask is agitated on a reciprocating shaker (90 rpm) for 15 minutes. Ten ml of 1:4 sulfuric acid are added and the mixture is titrated with 0.5 N potassium permanganate.

TABLE 1

Variation of replicate plate counts on replicate shaker cultures of Brucella suis

FLASK NO.	PLATE NUMBER										
	1	2	3	4	5	6	7	8	9	10	Mean
	Viable cells per ml × 10^{-9}										
1	52	42	49	51	56	45	55	61	55	55	52.1
2	37	53	54	51	47	61	48	44	43	62	50.0
3	47	50	51	58	51	43	44	54	50	56	50.4
4	53	59	54	53	64	54	54	39	33	62	52.5
5	58	48	43	51	52	37	47	30	54	51	45.3
6	61	48	58	60	52	52	48	54	52	46	53.1
7	55	49	46	62	53	62	56	56	44	56	53.9
8	56	61	52	49	57	74	53	55	53	61	57.1
9	72	57	52	66	67	95	71	58	64	68	67.0
10	52	53	48	47	58	50	53	51	56	53	52.1
Mean.......	53.5	52.0	50.7	54.8	54.7	57.3	52.9	50.2	50.4	57.0	53.4

Oxidation-reduction potentials were determined, using a shaker flask system of platinum electrodes, a saturated potassium chloride agar bridge to a calomel half cell, and a Beckman model G potentiometer. Three electrodes were used in each of three flasks, each determination thus being the average of nine replicate readings. The shaker was stopped when readings were taken.

The pH was determined directly from a shaker flask having a glass electrode, a saturated potassium chloride agar bridge, and a plugged inoculation tube in a diaphragm-type rubber stopper. The glass electrode had previously been standardized against a second electrode, so that the pH meter could be balanced against standard buffer solution without removing the culture electrode.

EXPERIMENTAL

Experiments were conducted to determine statistically the extent of variation of evaluations of plate count, light transmittance of the 1:10 dilution, and titration for catalase activity. Ten replicate 24-hour shaker flasks were used, each flask being analyzed by ten replications of each of three methods. The results are given in tables 1, 2, and 3. The variation between *replicate flasks* was found

TABLE 2

Variation of replicate light transmittance determinations on replicate shaker cultures of Brucella suis

FLASK NO.	DETERMINATION NUMBER										
	1	2	3	4	5	6	7	8	9	10	Mean
	Per cent light transmittance of 1:10 dilution										
1	18.0	18.2	18.0	18.0	18.8	18.0	18.2	18.0	18.0	19.0	18.0
2	19.2	18.2	19.0	18.8	18.0	18.2	18.8	18.5	19.2	18.2	18.5
3	19.8	18.8	18.8	18.8	19.0	19.0	19.5	19.0	19.2	18.8	19.0
4	18.0	19.0	18.5	19.0	18.5	18.8	18.8	18.5	19.0	19.5	18.8
5	19.2	19.8	20.0	20.0	18.8	19.2	19.0	18.8	19.0	19.0	19.2
6	19.8	20.0	20.2	19.5	20.0	19.0	20.0	19.8	19.2	19.2	19.8
7	18.2	18.0	19.0	18.5	18.5	18.0	18.5	18.0	18.0	18.8	18.2
8	19.5	19.0	19.5	18.5	19.8	20.0	19.2	19.5	20.0	20.0	19.5
9	18.0	18.2	18.5	18.8	19.0	19.0	18.2	18.2	18.2	18.8	18.5
10	18.2	19.0	18.2	18.0	18.5	18.0	18.8	18.5	19.0	18.5	18.5
Mean.......	18.8	18.8	19.0	18.8	18.8	18.8	19.0	18.8	19.0	19.0	18.8

TABLE 3

Variation of replicate catalase activity determinations on replicate shaker culture of Brucella suis

FLASK NO.	DETERMINATION NUMBER										
	1	2	3	4	5	6	7	8	9	10	Mean
	Catalase equivalent in ml of 0.5 N potassium permanganate										
1	26.2	26.7	27.7	26.7	26.1	25.9	25.8	25.6	25.7	25.6	26.20
2	25.7	25.2	26.7	24.8	24.2	25.1	24.4	24.7	24.3	24.7	24.98
3	24.2	23.7	25.9	24.3	24.3	23.7	24.3	24.1	23.8	23.3	24.16
4	25.4	25.5	26.3	26.0	25.3	25.2	25.2	25.2	25.1	25.7	25.49
5	26.0	25.0	25.3	24.6	23.6	24.1	25.1	23.5	24.2	24.4	24.58
6	24.6	25.1	26.4	25.4	25.4	24.7	25.3	24.1	24.3	25.2	25.05
7	25.6	26.3	25.6	24.6	23.6	23.4	23.3	23.5	23.2	24.0	24.31
8	25.2	24.8	25.8	25.4	24.8	25.4	25.2	24.4	25.1	24.8	25.09
9	26.6	24.8	25.3	25.1	24.6	24.1	24.6	23.9	24.1	24.4	24.75
10	26.8	25.9	26.3	26.7	25.4	25.2	26.6	24.9	24.5	25.1	25.74
Mean.......	25.63	25.30	26.13	25.36	24.73	24.68	24.98	24.39	24.43	24.72	25.04

to be significant for all variables; however, the relatively low values (10.87 per cent for plate count, 2.65 per cent for turbidity determination, and 2.49 per cent for catalase activity titration) obtained for the coefficient of variation indicated that this experimental variation was within allowable limits for biological work.

It was decided that duplicate flasks would be used for routine determinations, and at least four flasks for critical determinations. The variation between replicate plate count and turbidity determinations was found to be not significant. In the case of replicate catalase activity titrations, the variation was significant; however, it was shown that there was a regression in values with the time necessary to complete the replicate titrations. The low values (5.14 per cent for plate count, 0.53 per cent for turbidity determinations, and 2.23 per cent for catalase activity titration) obtained for coefficients of variation between *replicate determinations* indicated this variation to be well within allowable limits, even when the variation of the catalase activity titrations was shown to be significant. It was decided that at least triplicate plates would be used, and it appeared that single turbidity and catalase activity determinations were satisfactory for routine work.

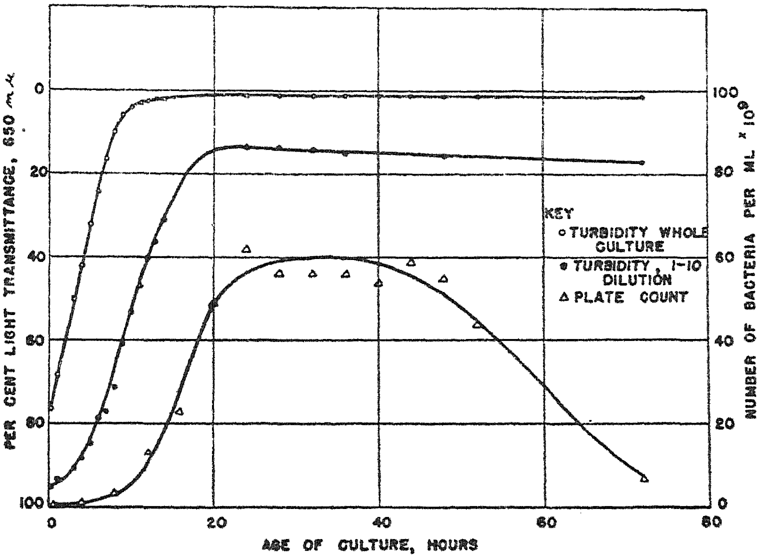

FIG. 3. PROGRESS OF GROWTH OF AERATED (SHAKER) CULTURE OF BRUCELLA SUIS IN FORTIFIED TRYPTOSE BROTH
Concurrent evaluations of light transmittance and plate count

The progress of growth of "normal," aerated (shaker) cultures in the aforementioned fortified tryptose broth was followed by concurrent evaluations of plate count, light transmittance of whole culture and 1:10 dilution, catalase activity, oxidation-reduction potential, and pH. Growth curves are given in figures 3, 4, and 5. These curves represent one experiment, with concurrent determinations. Several general observations were made. Use of this highly aerated (by shaking) system, together with a heavy inoculum of actively growing cells, apparently decreased the growth period from 50 to 70 hours to approximately 24 hours, minimized the lag phase of growth, and produced higher maximal counts. From these and similar results it appeared probable that evalua-

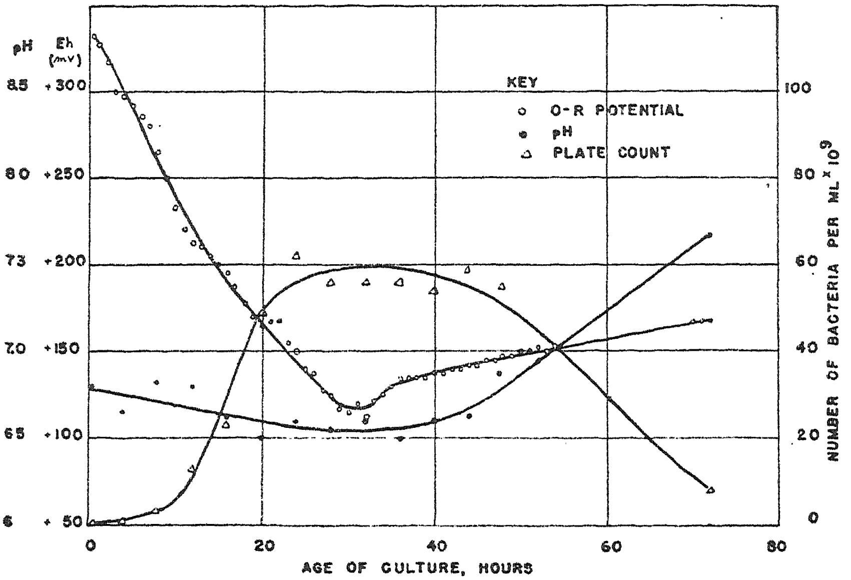

FIG. 4. PROGRESS OF GROWTH OF AERATED (SHAKER) CULTURE OF BRUCELLA SUIS IN FORTIFIED TRYPTOSE BROTH
Concurrent evaluations of oxidation-reduction potential, pH, and plate count

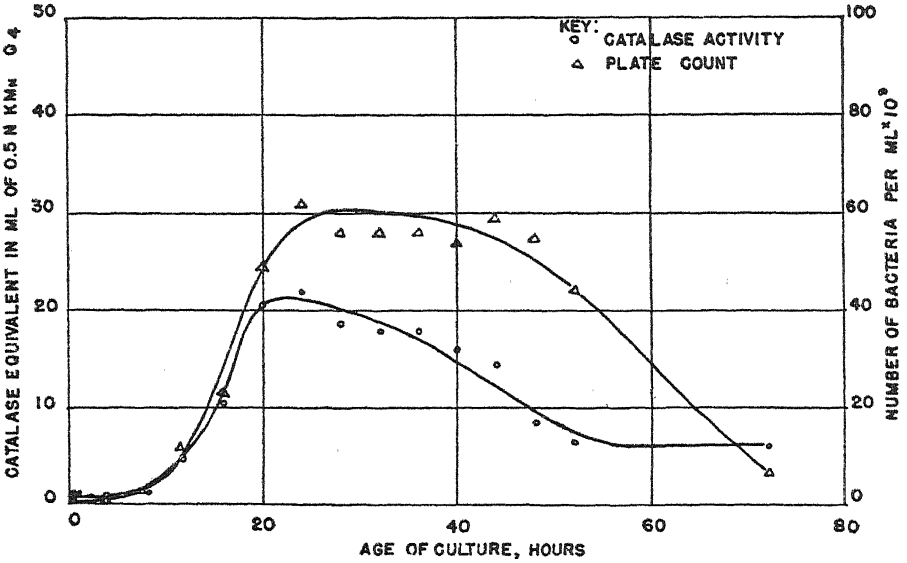

FIG. 5. PROGRESS OF GROWTH OF AERATED (SHAKER) CULTURE OF BRUCELLA SUIS IN FORTIFIED TRYPTOSE BROTH
Concurrent evaluations of catalase activity and plate count

tions of light transmittance, using the 1:10 dilution also employed for the plate count, may be utilized in larger scale production to indicate the peak of growth shortly before the actual peak of viable count. The catalase activity titration, used concurrently, may then provide a more directly correlated index for time of harvesting. Actual viable cell numbers, of course, may then be found some 2 to 4 days later, after plates have incubated. Although oxidation-reduction potentials may offer some evidence as to aeration efficiency and apparently are correlated somewhat with the increase in cell numbers, the technique involved possibly is too delicate and variable to be applied satisfactorily. Determinations of pH, of course, offer a simple control measure, valuable chiefly as an indirect method for determining contamination or irregularities in growth.

Past experience has dictated that control of yield, growth period, strain variation, etc., may often be accomplished by the employment of proper inocula. Consequently, experiments were conducted to determine the influence of the amount and age of inoculum upon the rate and extent of growth of the ensuing cultures. The results of such an experiment are given in figure 6. An inoculum culture was grown as usual on the shaker. Samples were removed after 16, 24, and 32 hours' incubation; the 24- and 32-hour cultures were diluted to equal the 16-hour count. The subcultures were inoculated with 0.01, 0.1, and 1.0 per cent by volume of the standardized inoculum of different ages. The progress of growth of these subcultures, as indicated by light transmittance of the 1:10 dilution, was then followed. Apparently there was a close correlation between the amount of inoculum used and the length of the lag phase; once the logarithmic phase was initiated, growth progressed at a similar rate. Thus, between the range of 0.01 and 1.0 per cent, a tenfold difference in the amount of inoculum manifested itself in approximately 8 hours' difference in the growth period. In every case, the maxima reached, as indicated by light transmittance and substantiated in another experiment by plate count, were approximately the same. Furthermore, it was indicated that the differences in the ages of inocula (taken

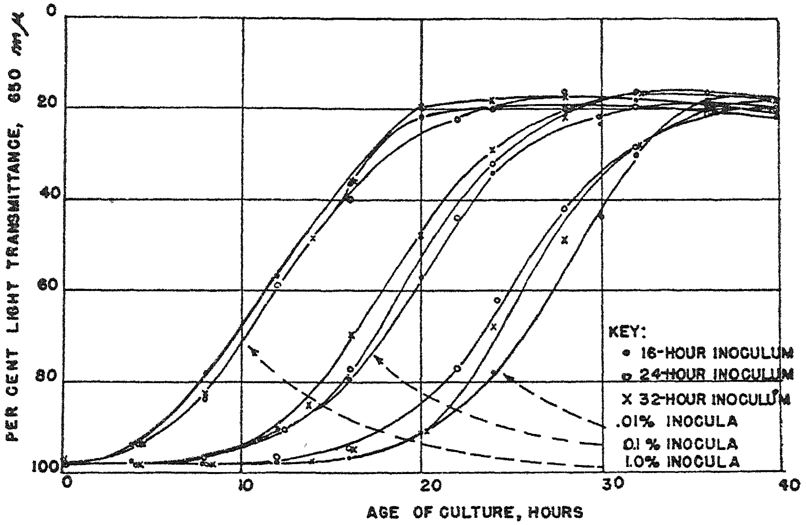

FIG. 6. INFLUENCE OF THE AMOUNT AND AGE OF INOCULUM UPON THE RATE AND EXTENT OF GROWTH OF AERATED (SHAKER) CULTURES OF BRUCELLA SUIS

approximately from the logarithmic phase, the phase of negative acceleration, and the maximum stationary phase) exerted no significant effect upon the rate or extent of growth. Thus, with the different amounts used there apparently was no consistent deviation due to differences in the age at which the inoculum was taken. It would be presumed, therefore, that it would be most economical in larger scale operations to utilize heavy inocula which had reached their maximal count. It was decided that an inoculum of 1.0 per cent by volume from a 24-hour shaker broth culture should be used as a routine, standard inoculum.

Several apparently variant strains were isolated through single colony selection from stock culture sources of the original strain of *Brucella suis*. In light of this observation, a more detailed study was conducted to determine whether strain variation occurred to an appreciable extent during routine handling of cultures. Consequently, a typical single-colony-derived culture was carried through 30 serial 24-hour transfers in fortified tryptose broth, plain tryptose broth, and on tryptose agar slants. No appreciable colonial variation nor any appreciable change in catalase activity was observed. In another study another typical single-colony-derived culture was carried through 63 serial transfers in the fortified tryptose broth, and it also failed to show any appreciable colonial variation.

DISCUSSION

Methods for plate count, pH, and oxidation-reduction potential determinations have been widely used and needed only to be applied to the requirements of this study. The catalase activity determination of Huddleson (Huddleson and Stahl, 1943) required some modification and standardization for routine use as an index of maximal growth. The technique was simple and gave results which correlated with the progress of growth of a culture, and could be used advantageously in conjunction with light transmittance to determine the point of maximal viable yield. Determination of light transmittance (turbidity) was modified to provide a convenient, accurate, and rapid assay of growth. Excellent results were obtained when the 1:10 dilution of the culture was employed. The cultures normally reached too low transmittance values for accurate evaluations of whole culture. Finally, statistical analyses were made of important assays to determine the extent of variation and the number of replications needed for normal accuracy.

Growth curves were then made under optimum conditions of substrate and aeration, using the foregoing assays concurrently. These curves served as references for subsequent experimental work, as the conditions under which the cultures were grown could be accurately reproduced. They served also as the basis on which determinations were subsequently made after a 24-hour growth period; it was indicated that comparisons could be made at this time with a fair degree of validity. Moreover, these growth curves suggested that light transmittance evaluations of the 1:10 dilutions of cultures could be used satisfactorily to indicate rapidly the peak of growth so as to determine the time for harvesting cultures. It was observed upon continued use that cultures could be safely harvested approximately 6 hours after the peak of light transmittance was reached in order to obtain a maximum count in the product.

Inoculum studies were undertaken as a means of control as well as to devise a method for increasing yields. The influence of the age and amount of inocula upon the rate and extent of growth of the ensuing cultures was determined. Relatively large amounts of inocula were employed in order to decrease the growth period and thus increase efficiency. Of particular note was the observation that the amount of inoculum, within the range employed, manifested itself primarily in the length of the lag phase, whereas the logarithmic rate and extent of growth remained virtually unaffected. Moreover, the age of the culture from which the inoculum was prepared, within the limits studied, was apparently of no influence in these respects. Insofar as routine handling of cultures, as well as the normal mass culture procedures, involved prolonged serial transfer of the organism, it was necessary to determine whether strain variation occurred to an appreciable extent under such circumstances. This factor assumed even greater significance in subsequent continuous culture studies. Although it was possible to isolate several apparently variant strains through single colony selection, colonial variation or change in catalase activity was present only to a limited and apparently unimportant degree when single-colony-derived "typical" cultures were carried for extended periods in serial transfer.

SUMMARY

Techniques for the determination of light transmittance, catalase activity, oxidation-reduction potential, pH, and plate count in broth cultures of *Brucella suis* were surveyed and modified. Statistical analyses were made of important assays.

Growth curves were established for shaker cultures in fortified tryptose broth, using concurrent evaluations of the foregoing assays.

Employment of a heavy inoculum of actively growing cells together with a highly aerated (shaker) system markedly decreased the growth period of the organism in broth culture and greatly increased yields.

Differences in the amount of inocula, within the range employed, manifested themselves primarily in the length of the growth period, but the rate and extent of growth remained virtually unaffected. Differences in the age of the culture from which inocula were prepared, within the limits studied, apparently were of no significant effect in these respects.

Single-colony-derived cultures of the organism failed to show any appreciable colonial variation or change in catalase activity when carried for an extended period in serial transfer.

ACKNOWLEDGMENT

The authors wish to express their deep appreciation for the excellent technical assistance of PhM3/c Elizabeth McIntyre, WAVE, and T/4 J. Czarnecki, Chemical Warfare Service. Dr. J. L. Roberts and Major S. S. Elberg, Sanitary Corps, supervised the study.

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BRUCELLA SUIS IN AERATED BROTH CULTURE

II. Aeration Studies[1]

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Resolution of the aeration requirements of *Brucella suis* in broth culture was imperative for the production of the comparatively large amounts of cells required in the extensive epidemiological and immunological project of which this study is a part. The two prime factors in sparger aeration appeared to be (1) the rate of air supply and (2) the type of sparger employed; the two factors apparently were interdependent. Thus, the more efficient the sparger in producing fine bubbles, in general the less air required for growth. This has been emphasized in a review of biological aeration systems by De Becge and Liebmann (1944). In the case of this organism, however, the kinds of antifoam employed with various sparger aeration systems has also been found to be of primary importance.

A typical industrial type stainless steel "dishpan" sparger has 0.108-inch perforations, 49 per square inch; whereas, in contrast, one of the more efficient industrial type heat-resistant carbon spargers (grade no. 60) has an average porosity of 0.0013 inches. Consequently, a detailed study was undertaken to compare air requirements, using different sparger types along with different antifoams. If through improved aeration air requirements could be lessened while high yields were maintained, the opportunity for contamination would be reduced, and foaming of the medium decreased. Additional studies were concerned with the employment of a gradually increasing air supply, the influence of the pressure head in the system, and the value of shaking as an aeration expedient.

METHODS

The following three types of aeration systems were employed in these studies:

(1) Shaker flasks, as reported previously (Gerhardt and Gee, 1946), in which a high, constant, and easily reproducible rate of air supply was obtained by growing the organism in flasks incubated on a shaking machine, oscillating horizontally a distance of 3.0 inches at a rate of approximately 90 oscillations per minute. Normally, 250-ml Erlenmeyer flasks were used, containing 25 ml of medium each.

(2) Aeration bottles, consisting of 1-quart jars, each fitted with a rubber stopper and a sparger. Normally, 400 ml of medium were placed in each bottle. Air was supplied by maintaining a negative pressure above the medium.

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[1] Studies conducted at Camp Detrick, Frederick, Maryland, January to September, 1945.

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(3) A column-type aeration system was also devised (figure 1), in which 4-foot sections of 80-mm pyrex tubing were modified so as to contain a sparger and sampling tube. The medium (3,250 ml) used in the apparatus provided a height of liquid of 30 inches. Air was supplied by maintaining a negative pressure above the medium.

Four types of spargers were employed: coarse grade sintered glass type, no. 60 grade carbon type (National Carbon Co., Cleveland, Ohio), improvised cotton muslin type, and perforated stainless steel "dishpan" type. Normally, the sintered glass spargers were employed with the aeration bottles, and the perforated stainless steel spargers with the column-type apparatus. The following medium was used: 2.0 per cent bacto tryptose (Difco), 0.75 or 1.0 per cent glucose, 0.5 per cent sodium chloride, 0.1 μg thiamine hydrochloride per ml (McCullough *et al.*, 1945) in distilled water. A single strain of *Brucella suis* was used throughout (received through the courtesy of Dr. I. F. Huddleson, his strain no. 1772-A). Normally, an inoculum of 1 per cent by volume from a 24-hour shaker broth culture was used; this was approximately 5 × 10^8 cells per ml final concentration. Incubation was at 37 C. Light transmittance was determined by use of the Coleman universal spectrophotometer on 1:10 dilutions of the culture. Unless otherwise stated, the figures in tables and graphs represent the average of triplicate 24-hour cultures.

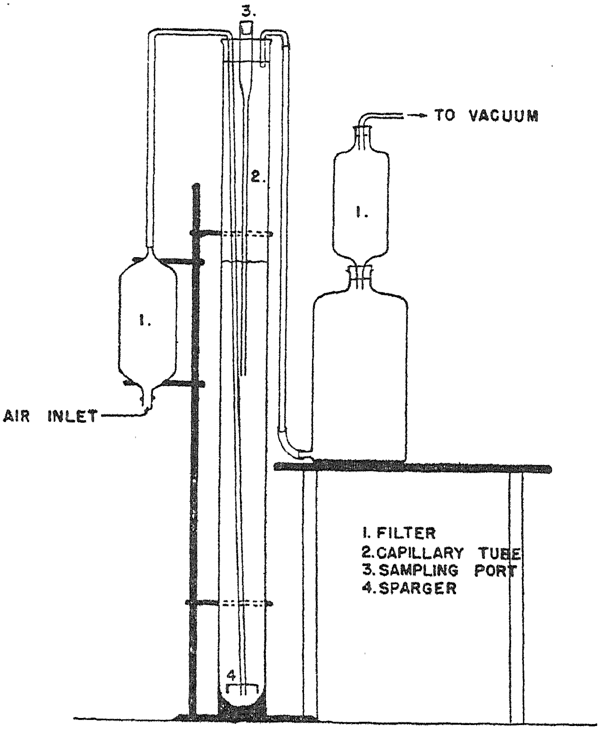

FIG. 1. COLUMN-TYPE AERATION APPARATUS

The aeration rates are reported in volume units per minute. For example, an aeration rate of one volume per minute means the passing through the system each minute of a volume of air equivalent to the volume of the medium. The aeration rate was controlled by varying the effective negative pressure. The air flow was measured with carefully calibrated rotameters, at atmospheric pressure.

Before the studies of sparger aeration systems, it was necessary to find a suitable antifoam agent for use with tryptose media under the conditions of violent agitation inherent in sparger aeration. Previous laboratory studies under somewhat different conditions had indicated the use of a rather heavy layer of pure lard. In a series of preliminary tests, approximately 15 different antifoam agents were screened for physical properties; of these, only a few warranted further study. The following agents were found unsatisfactory: capryl alcohol, Atlas "arlacelc," turkey red oil, corn oil, DuPont "ocenol," octyl alcohol, lard oil, lard oil and octadecanol, lard oil and tributyl citrate, and olive oil. DuPont HF and LF antifoams were found to have excellent physical properties, but subsequently they were found to be highly toxic to *Brucella suis*. A 50:50 mixture of lard and tributyl citrate had good antifoam properties and was only partially toxic. Pure lard had satisfactory antifoam properties under certain conditions and apparently was somewhat toxic only under shaker conditions; the extreme turbulence effected by shaker aeration apparently made such tests not entirely applicable to sparger aeration conditions. Unless otherwise stated, an equal mixture of lard and tributyl citrate was used as the antifoam agent.

EXPERIMENTAL

Shaker flasks. Since the shaker-flask culture was used as a standard, it was considered advisable to alter the ratio of the medium volume to the flask volume in order to determine optimum conditions for a maximum cell count of the standard medium. This was done by placing 25 ml, 50 ml, 100 ml, and 200 ml of medium in 250-ml, 500-ml, and 1,000-ml Erlenmeyer flasks. The results of this experiment are given in table 1. From this experiment and the extensive amount of confirmatory evidence accumulated from other shaker-grown cultures, it was concluded that the standard practice of using 25 ml of medium in a 250-ml Erlenmeyer flask provided near optimum aeration and could be used as a convenient standard reference procedure.

Aeration bottles. The preliminary study on the type of sparger and the aeration rate required for maximal growth of *Brucella suis* was conducted in aeration bottles. Table 2 gives a compilation of data from a number of experiments designed to compare various rates of aeration with three different types of spargers. Under the conditions of comparison, the fine dispersion afforded by sintered glass, and even plain cotton muslin, was apparently considerably more efficient in promoting growth of *Brucella suis* than the coarser aeration of the stainless steel "dishpan" type. The sintered glass type apparently provided optimum aeration at approximately one volume of air per minute; quantities in excess of this apparently caused increases in cell numbers only through evaporation.

A study was made to determine whether a gradual build-up of air supply could be substituted for the sustained aeration rate normally employed. This system would present two advantages if successful: (1) reduction of the total air requirements and consequently of the possibilities of contamination, particularly

TABLE 1

The influence of amount of medium and size of Erlenmeyer flask on shaker-grown cultures of Brucella suis

FLASK SIZE	AMOUNT OF MEDIUM	LIGHT TRANSMITTANCE	VIABLE CELLS × 10^{-9}
ml	*% ml*	*ml*	*per ml*
250	25	15.3	50
	50	16.8	53
	100	33.4	32
	200	82.3	4
500	25	12.8	55
	50	15.7	47
	100	19.5	48
	200	38.9	28
1,000	25	13.6	46
	50	15.0	53
	100	18.1	46
	200	19.0	39

TABLE 2

The influence of types of spargers and aeration rates on the growth of Brucella suis in aeration bottles

EXP. NO.	TYPE OF SPARGER	AERATION RATE	LIGHT TRANSMITTANCE	VIABLE CELLS × 10^{-9}	
				Actual count	Corrected count*
		vol/min	*%*	*per ml*	*per ml*
1	Sintered glass	0.1	59.7	5.7	
1	Sintered glass	1.0	18.0	37.7	
2	Sintered glass	1.0	17.0	41.7	
2	Stainless steel	1.0	77.0	0.6	
3	Sintered glass	1.0	22.0	39.0	
3	Stainless steel	5.0	65.0	1.6	
18a	Stainless steel	10.0	28.0	77.0	36.0
6	Sintered glass	1.0	16.5	57.3	52.0
7	Sintered glass	3.0	12.5	62.2	53.5
8	Sintered glass	6.0	9.0	73.7	42.5
31a	Sintered glass	1.0	19.0	54.0	
31a	Cotton muslin	1.0	34.0	16.0	
32a	Sintered glass	1.0	21.0	39.0	
32a	Cotton muslin	3.0	15.5	46.0	

* Count corrected to compensate for evaporation.

in early stages of growth; (2) reduction of foaming in the early stages of growth, when it is most difficult to control. A series of experiments was conducted in which various schedules of increasing air rates were compared with a constant high air rate. The results are given in table 3. They indicate that a gradually increasing air rate might be employed to advantage, although no increases in yield were obtained.

TABLE 3

Comparison of graduated air supply with constant air supply on the growth of Brucella suis in aeration bottles

EXP. NO.	AERATION RATE AND SCHEDULE*	LIGHT TRANSMITTANCE	VIABLE CELLS × 10^{-9}
	vol/min	%	*per ml*
24	0.1–1.0(8)	20.0	46
	1.0	17.5	50
26	0.1–0.25(4)–0.5(6)–1.0(8)	17.5	44
	1.0	18.0	40
27	0.1–0.25(4)–0.5(6)–1.0(12)	17.0	43
	1.0	16.5	43
30	0.25–0.5(5)–1.0(12)–3.0(18)	19.5	43
	1.0	16.5	43

* Figures in parenthesis indicate the age of culture in hours when the aeration rate was changed.

TABLE 4

Comparison of two types of spargers on the growth of Brucella suis in the column-type aeration apparatus

EXP. NO.	TYPE OF SPARGER	AERATION RATE	LIGHT TRANSMITTANCE	VIABLE CELLS × 10^{-9}
		vol/min	%	*per ml*
2	Carbon	0.5	29.5	14.0
	Stainless steel	0.5	65.0	2.5
3	Carbon	0.25	25.0	24.0
	Stainless steel	2.0	50.0	6.4
5	Carbon	0.25	23.0	22.7
	Stainless steel	4.0	62.0	4.1

Column-type aeration apparatus. The comparison of sparger types was further studied in the column-type aeration apparatus. The study was restricted to a comparison of the perforated stainless steel "dishpan" type with the grade no. 60 carbon sparger. The results given in table 4 show that under the conditions of the comparison, the finer air dispersion of the carbon sparger was again much more efficient in promoting growth of *Brucella suis* than the coarser aeration afforded by the stainless steel units. Thus, the maximum air supply possible in the apparatus with the perforated stainless steel sparger (four volumes per minute) produced less growth than did one-quarter volume per minute with the carbon sparger. However, it was felt that the slight toxicity of the lard tributyl citrate antifoam noted previously might be greatly enhanced by the extreme turbulence present in the columns aerated with the stainless steel sparger. A differential toxicity of the antifoam would, of course, invalidate the comparison of the sparger types. Consequently a test of the toxicity of lard and lard tributyl citrate was made in the column-type aeration apparatus using the stainless steel spargers. The results, given in table 5, show that under conditions of high turbulence the lard tributyl citrate antifoam was highly toxic. Thus, it appeared possible that the apparent superiority of finer spargers (such as the grade no. 60 carbon type), which require lard and tributyl citrate in order to control foaming effectively at the higher aeration rates, might be considerably reduced or surpassed when compared to perforated stainless steel spargers which require only the nontoxic lard in order to control foaming effectively. The two types of spargers were, therefore, compared, pure lard being used as antifoam. The aeration with the carbon spargers was maintained at the highest rate possible without foaming over (increasing from 0.125 to 0.5 volumes per minute), whereas the aeration with perforated stainless steel spargers was maintained at an optimum rate of two volumes per minute. In this study (table 6) practically identical yields were obtained. It would appear, therefore, that lard alone was feasible as an antifoam with coarse spargers, or with fine spargers at relatively low aeration rates. It was also evident that a fine dispersion of the air was most efficient, but increased the difficulties encountered with foaming.

TABLE 5

*The influence of lard and lard tributyl citrate antifoams on the growth of Brucella suis in the column-type aeration apparatus**

EXP. NO.	ANTIFOAM ADDED	LIGHT TRANSMITTANCE	VIABLE CELLS × 10^{-8}
		%	*per ml*
6	None	20.0	46.5
	3 ml lard	16.5	55.5
	3 ml lard + TBC	31.0	19.5
7	None	18.0	40.0
	3 ml lard	18.5	47.5
	3 ml lard + TBC	52.0	5.5

* Aerated at the maximum rate obtainable with no antifoam, 1 to 2 volumes per minute, with perforated stainless steel spargers.

Composite results of a series of experiments showing the influence of various aeration rates upon the over-all growth picture of *Brucella suis* are given in figure 2. Aeration at about 2.0 volumes of air per minute gave the highest

TABLE 6

Comparison of perforated stainless steel and grade 60 carbon spargers on the growth of Brucella suis in the column-type aeration apparatus using lard antifoam

TIME	CARBON SPARGER		STAINLESS STEEL SPARGER	
	Aeration* rate	Viable cells × 10^{-9}	Aeration rate	Viable cells × 10^{-9}
hrs	*vol/min*	*per ml*	*vol/min*	*per ml*
0	0.125	0.49	2.0	0.63
8	0.25	1.44	2.0	1.14
12	0.5	5.7	2.0	4.0
16	0.5	13.3	2.0	12.0
20	0.5	27.3	2.0	23.3
24	0.5	38.0	2.0	34.3
28	0.5	47.3	2.0	46.0
32	0.5	48.0	2.0	50.7
36	0.5	53.0	2.0	54.3

* Maintained at a maximum for the carbon sparger, lard system.

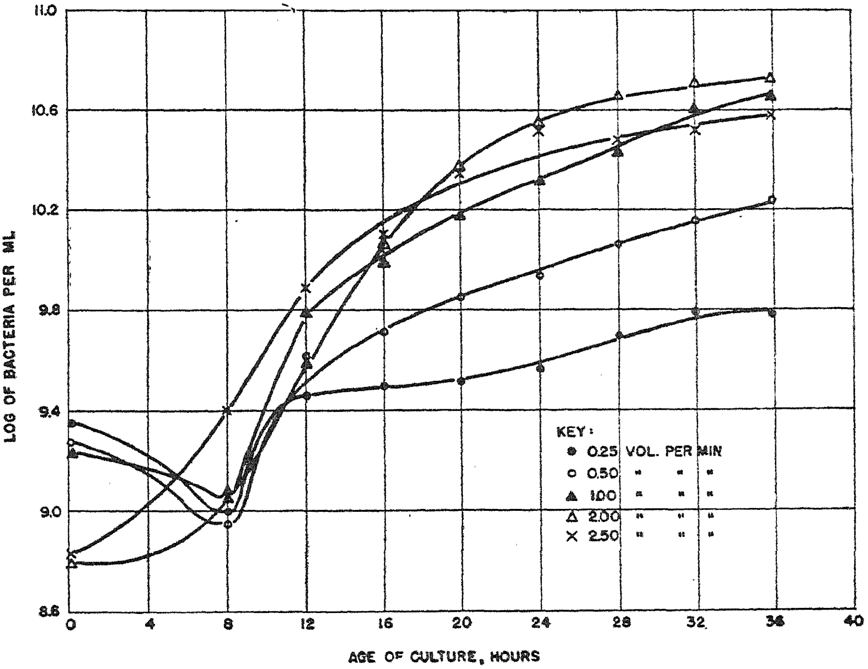

FIG. 2. INFLUENCE OF AERATION RATES ON THE GROWTH OF BRUCELLA SUIS IN COLUMN-TYPE AERATION APPARATUS

yield, and a rapid rate of increase in the logarithmic phase. Although 2.5 volumes of air per minute gave better initial growth, the rate of increase and the maximum yield were slightly less. At 1.0 volume per minute aeration, the rate of increase was somewhat slower, but the final yield at 60 hours actually equaled that with 2.0 volumes per minute (results not given). Aeration rates of 1.0 volume and less per minute were apparently suboptimal for maximum rate of growth. It was concluded, therefore, that approximately 2.0 volumes of air per minute was optimal for the growth of *Brucella suis* in this apparatus.

It was desirable to have a single figure that would express the comparative influence of an aeration rate upon the growth of *Brucella suis* in the column-type aeration apparatus. For this purpose, the viable cell count generation times were calculated from the constant aeration rate data. These generation times are given in table 7. The growth period from 12 to 16 hours was found to be most representative. The results show that the generation time was sharply shortened as the aeration rate was increased up to 2.0 volumes per minute. The reverting rise at 2.5 volumes per minute can only be taken as an indication that with this system aeration rates above 2.0 volumes per minute are not necessary for optimal growth. This observation is further strengthened by the results obtained with the built-up aeration rates reported in table 8.

TABLE 7

The viable cell count generation time of Brucella suis grown in the column-type aeration apparatus using different aeration rates

EXP. NO.	AERATION RATE	VIABLE CELLS × 10^{-9}		GENERATION TIME
		12 hr	16 hr	
	vol/min	*per ml*	*per ml*	*hrs*
11	0.25	2.90	3.17	31.1
	0.5	4.05	5.10	12.0
	1.0	6.35	10.0	6.1
8	2.0	4.0	12.0	2.5
9	2.0	5.1	15.0	2.6
12	2.0	1.3	3.8	2.6
13	2.5	7.7	12.5	5.7

The study on the gradual building up of the air supply was conducted in somewhat more detail with the column-type aeration apparatus than with the aeration bottles. Composite results of this study are given in table 8. Although no definite increase in yield or rate of growth was obtained, again a gradual increase of the air supply accomplished results comparable to those obtained with a constant aeration rate. Thus, the air requirements were considerably lowered, and the initially lower aeration rates served to diminish foaming at the most critical foaming period.

All work with sparger aeration apparatus has been operated entirely under negative pressure as a safety factor in the production of comparatively large amounts of *Brucella suis* cells. It was noted early in the aeration study that

TABLE 8

Comparison of graduated air supply with constant air supply on the growth of Brucella suis in column-type aeration apparatus

EXP. NO.	AERATION RATE AND SCHEDULE*	VIABLE CELLS PER ML × 10^{-9}								
		0 hr	8 hr	12 hr	16 hr	20 hr	24 hr	28 hr	32 hr	36 hr
	vol/min									
9	0, ⅛(4), ¼(8), ½(12), 1(16), 2(20)	1.6	0.6	2.9	4.4	10.1	20.0	29.0	47.0	60.0
	¼(4), ½(8), 1(12), 2(16)	2.0	1.3	4.0	10.3	16.5	33.0	39.6	50.0	57.0
	2	1.9	1.1	5.1	15.0	21.5	32.0	42.5	55.0	51.5
10	1, 2(8), 4(12)	1.6	2.2	6.1	10.5	25.0	33.0	44.0	63.0	46.5
	1, 2(8)	2.4	2.0		16.0	30.0	33.0	55.0	61.0	53.0
	1	1.9	2.1	5.9	16.0	16.0	25.5	31.0	38.0	42.5
12	1, 2(12)	0.3	0.5	2.8	4.9	10.1	14.5	19.0	32.5	43.5
	2	0.3	0.4	1.3	3.8	9.4	16.5	23.3	31.3	44.0
13	1, 1½(12), 2(16), 2½(20)	0.4	2.9	11.7	12.5	21.0	31.0	32.5	33.0	33.0
	1, 1½(8), 2(12), 2½(16)	0.5	3.3	11.5	13.5	27.0	37.0	37.0	34.0	36.5
	2½	0.7	2.5	7.7	12.5	23.5	34.0	30.5	33.0	38.5
15	1, 2(12), 3(16), 4(20)	1.7	1.8	6.1	13.0	30.0	30.5	42.0	55.0	49.5
	1, 2(12), 3(16)	1.0	1.9	5.7	12.5	32.0	37.5	52.0	47.5	45.5
	1, 2(12)	0.9	2.4	6.4	15.5	27.0	29.0	41.0	36.5	43.5

* Figures in parenthesis indicate the age of the culture when the aeration rate was changed.

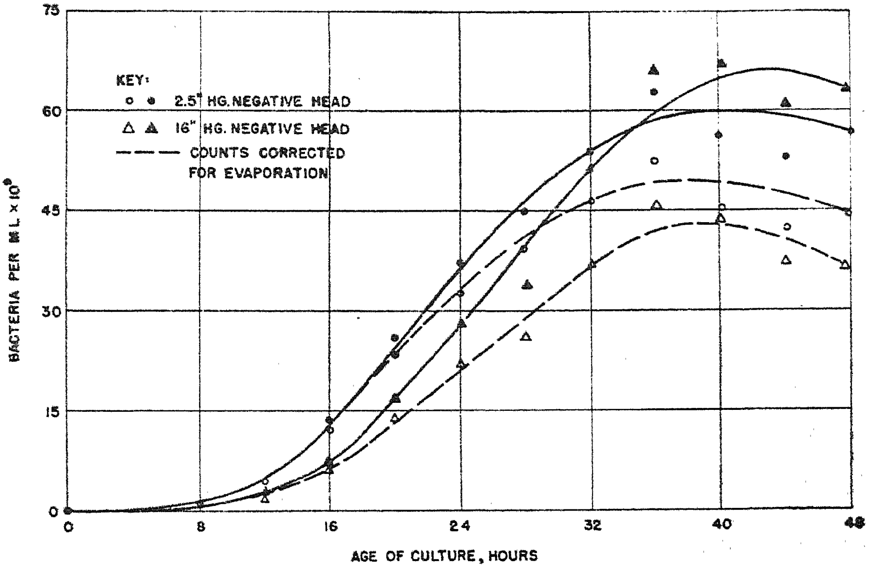

FIG. 3. INFLUENCE OF NEGATIVE PRESSURE HEAD ON THE GROWTH OF BRUCELLA SUIS IN THE COLUMN-TYPE AERATION APPARATUS

many factors (e.g., the porosity of the filters, the height of the column of liquid, the size of the tubing, the porosity of the spargers) markedly influenced the amount of the negative head above the medium. Since the solubility of a gas is a function of the pressure of the gas above the liquid, it was felt that this factor had to be evaluated in this aeration study. Consequently, an experiment was designed to compare the effect of two widely different negative pressure heads upon the growth of cultures in the systems, the two having an equal air supply as measured at atmospheric pressure. The results are given in figure 3. One effect of the lower pressure was to increase the amount of evaporation almost twofold. Consequently, valid interpretation of the results necessitated correction of the final counts to compensate for this evaporation. It then became apparent that, rather than the wide differences anticipated, the two systems gave approximately the same results. It would appear, therefore, that within the range normally encountered in the laboratory the negative pressure head exerted little effect on the sparger aeration systems operating at optimum aeration rate.

DISCUSSION

Aeration is one of the most important factors in the production of large amounts of *Brucella suis* cells. With the sparger type aeration systems used in this study, foaming of the medium presented an additional problem. Consequently, it was necessary to conduct a preliminary study to find an antifoam agent that would control foaming and at the same time be nontoxic to the organism. A screening test of a number of such agents resulted in the selection of pure lard and an equal mixture of lard and tributyl citrate as the most feasible. Satisfactory results were obtained in aeration bottle studies using the lard citrate antifoam, in which only this agent would adequately control foaming with the fine sintered glass spargers. However, relatively low yields were obtained in the column-type apparatus when this antifoam was employed, regardless of the sparger type. This result apparently was due to the excessive turbulence of the system. With pure lard as an antifoam, however, good results were obtained with either fine carbon spargers at their maximum attainable rate (approximately 0.5 volumes) or the coarse, perforated, stainless steel spargers at their optimum rate (approximately 2.0 volumes). Thus, the lard antifoam, carbon sparger system was four times as efficient in air requirements as the lard antifoam, perforated stainless steel sparger system. The latter, in turn, gave higher yields than either sparger system with lard citrate antifoam.

The viable cell count generation time, as well as the maximum yields, was used as an index of aeration efficiency. It was found that the generation time between 12 and 16 hours was the most representative of an aeration rate. With the stainless steel spargers, which were used because of ease of control and latitude in aeration rates, the generation time was reduced from 31.1 hours with an aeration rate of one-quarter volume per minute to 2.6 hours at an aeration rate of 2.0 volumes per minute. Two volumes per minute aeration was the optimum rate for this system.

The influence of the negative pressure head in the aeration systems upon the efficiency of aeration, and consequently the growth of the culture, appeared to be minimal within the range normally encountered in the laboratory.

A gradual building up of the air supply during growth accomplished substantially the same results as those obtained with a constant high aeration rate. It serves to reduce the total air requirements, thereby minimizing the possibilities of contamination, particularly in the early stages of growth, and to reduce foaming at the time when it is most difficult to control.

SUMMARY

Employment of a reciprocating shaker apparatus provided an optimum, yet constant and easily reproducible, rate of air supply for broth cultures of *Brucella suis*. This technique was used as a standard reference procedure.

Aeration requirements for maximum growth of the organism in sparger aeration systems were interdependent upon two factors: type of sparger and the antifoam agent necessary to control foaming. Although an equal mixture of lard and tributyl citrate was sometimes necessary to control foaming with spargers of fine porosity, it became toxic under conditions of high turbulence. An antifoam of pure lard was nontoxic under most conditions and satisfactorily controlled foaming with coarse spargers or with fine spargers at low aeration rates. Fine, grade no. 60, carbon spargers gave comparably high yields with approximately one-fourth the air requirements of coarse, perforated, stainless steel spargers.

The viable cell count generation time was used as an index of aeration requirements. This generation time changed from 31.1 hours to 2.6 hours with an aeration change from one-quarter volume to 2.0 volumes per minute.

There was no appreciable difference between the effects produced by negative pressure heads of 2.5 and 16 inches of mercury on aeration efficiency at an aeration rate of 2 volumes per minute.

The employment of a gradually increasing aeration rate gave approximately the same yields as a constant high aeration rate and enhanced the control of foaming in the early stages of growth of sparger-aerated cultures.

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BRUCELLA SUIS IN AERATED BROTH CULTURE

III. Continuous Culture Studies[1]

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The maintenance of a given bacterial population by the continuous addition of fresh medium and the withdrawal of culture presumably represents the ultimate in efficiency for the production of the comparatively large amounts of organisms that are normally required for immunochemical investigations. The rapid growth, nonsporulation, apparently stable strain characteristics, and aerobic nature of *Brucella suis* made it seem particularly adaptable to such a process. Consequently, this study was undertaken to devise a laboratory process for continuous culture of the organism that might be translated to the production of larger quantities for chemical and immunological studies.

The literature contains several reports regarding similar techniques, although none was directly applicable. A continuous vinegar fermentation process has been described and applied industrially, but it seemed to have limited usefulness in this study. Similarly, laboratory apparatus and experimental uses have been described by several workers (Moyer, 1929; Haddon, 1928), but in each case the method was not adapted to the requirements of this investigation. A system originated by Rogers (Rogers and Whittier, 1930) and adapted by Cleary (Cleary, Beard, and Clifton, 1935) suggested a means of attaining the results desired. Modifications were necessary, however, for its use with a highly pathogenic organism, for more accurate flow control, and for carefully controlled aeration. Probably the most applicable studies have been reported by Kolachov and coworkers (Unger *et al.*, 1942; Bilford *et al.*, 1942), who have developed an apparently successful continuous aerobic process for the production of distillers' yeast on a pilot plant scale. They were able to reduce the allover processing time from 20 to 40 hours to 4 hours for an equivalent volume of product, at the same time increasing yields from 150,000,000 to 500,000,000 cells per ml and obtaining yeast of high quality, uniformity, purity, and fermentability. It is to be noted, however, that in no case reported in the literature were highly virulent organisms employed extensively.

APPARATUS AND METHODS

Preliminary experiments on the regeneration time (i.e., the time necessary to reproduce a given population) and growth characteristics of the organism were conducted in shaker flasks, but subsequent studies employed modifications of aeration bottles (Gee and Gerhardt, 1946). In the first technique, cultures were grown in 25 ml of medium in a 250-ml Erlenmeyer flask, and aerated by continuous agitation on a reciprocating shaker at approximately 90 rpm; by this method a high, constant, and easily reproducible rate of air supply was obtained, providing accurately controlled cultural conditions. In the second technique, ordinary 1-quart jars were fitted with a rubber stopper and a coarse grade sintered glass sparger and partially filled with 400 ml of medium; filtered air was supplied by negative pressure. The following medium (McCullough *et al.*, 1945) was used: 2 per cent bacto tryptose, 0.5 per cent sodium chloride, 0.75 or 1.0 per cent glucose, 0.1 μg thiamine hydrochloride per ml, and distilled water. A single strain of *Brucella suis* was employed throughout (received through the courtesy of Dr. I. F. Huddleson, his strain no. 1772-A). Incubation was at 37 C. Plate counts were made by the usual poured plate methods using tryptose agar (Difco). Light transmittance was determined by use of the Coleman universal spectrophotometer, using matched 18-mm pyrex test tubes as cuvettes. Evaluations were made on the 1:10 dilution of the culture, using a 1:10 dilution of the original medium as the reference standard.

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Initially, two basic concepts of continuous culture were considered: (1) a so-called "cyclic" continuous system, in which the addition of fresh medium and the withdrawal of culture were made at periodic intervals, a portion of the product being retained as starter for the ensuing cycle; and (2) a completely continuous system, in which the addition of fresh medium and the withdrawal of product were accomplished continuously and simultaneously. A description of the laboratory apparatus devised for these systems follows:

(1) The "cyclic" system was applied to either one or a number of culture vessels in series. When operated as a unit, the culture was grown for a given period of time, at which point 50 or 90 per cent of the product was withdrawn to a receiving flask, an equivalent amount of fresh sterile medium was added to the vessel, and the culture was allowed to regenerate again to the given level to complete the cycle. Fundamentally, this amounted to a serial transfer in a closed system employing large inocula. The same system, operated with a number of vessels, was so arranged as to employ one vessel to provide inoculum for two or more culture vessels; for instance, the inoculum vessel might regenerate in a 4-hour cycle to balance three culture vessels, each maintaining a 12-hour cycle. Either modification of the cyclic system was operated in a completely closed apparatus; addition of medium, withdrawal of culture, and aeration were accomplished by negative pressure through manifold flow tubes.

(2) A schematic diagram of the apparatus devised for the completely continuous system is presented in figure 1. A supply of sterile medium was contained in the storage flask and was replenished infrequently by aseptic addition through a sampling tube. The medium was drawn from this flask to the culture vessel through capillary tubing and a standard orifice to regulate the supply. The change in flow that would be encountered as the supply of medium was depleted (due to the change in hydrostatic pressure) was obviated by the compensating effect of the air inlet capillary and the medium outlet capillary reaching

to the bottom of the flask. In the culture vessel, a given population was maintained, depending upon the rate of medium supply and product removal. This rate of operation was regulated by the capillary and standard orifice restrictions and by the amount of negative pressure. Air was supplied at a constant and previously determined optimal rate of 400 ml per minute. Foaming was controlled by the addition of an equal mixture of lard and tributyl citrate. Samples were withdrawn through the sampling tube. Twenty-four-hour intervals were allowed for the system to reach equilibrium. Culture was withdrawn through the vacuum line, maintaining a constant level of 400 ml in the reactor. The rate of product withdrawal (and therefore medium supply as well) was measured by by-passing the effluent into the meter and timing the passage of a given amount. The product was collected in a large receiving flask. A constant vacuum was maintained in the system by the use of a portable $\frac{1}{8}$ HP rotary pump. Sterilization of the air entering the culture and medium storage vessels, as well as the effluent air, was effected by cotton filters.

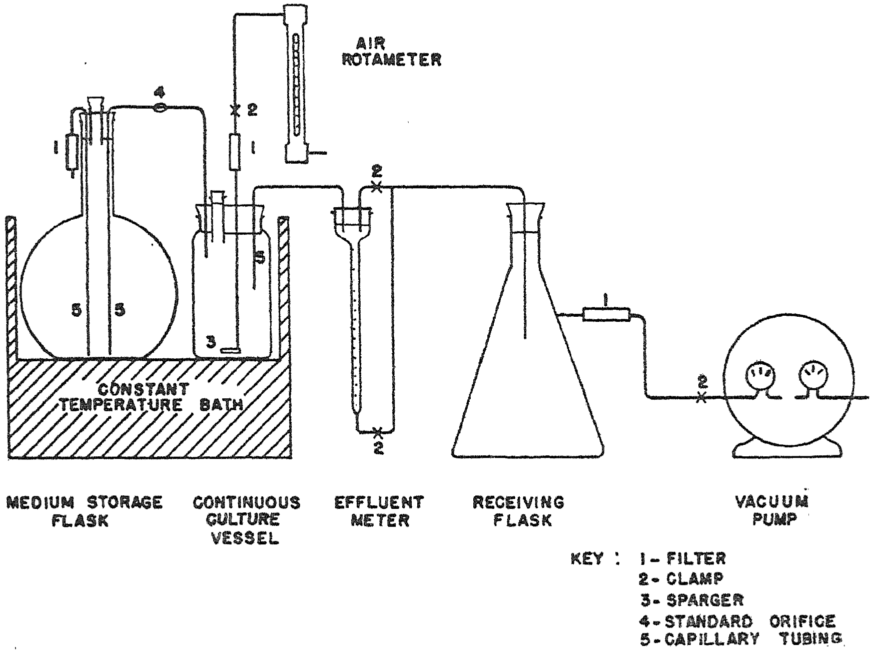

FIG. 1. DIAGRAM OF COMPLETELY CONTINUOUS CULTURE APPARATUS

The efficiency of operation of the various systems was expressed as the increase in cell count per hour. Although calculated somewhat differently for each system, the various efficiency data are on a comparable basis.

EXPERIMENTAL

Preliminary experiments were conducted to determine the time necessary for the regeneration of cultures, using different levels of inocula taken from various periods in the growth phase. Thus, samples were removed from a parent culture when it had reached light transmittance levels of 40, 30, and 20 per cent, and were used to inoculate subcultures; the time necessary to regenerate these levels was then determined. Theoretically, a cyclic continuous system would merely represent a continued repetition of this process. Composite results of such an experiment are given in table 1. Although a 10 per cent inoculum level apparently gave a somewhat comparable efficiency of production, the 50 per cent level was used in subsequent work by reason of its more ready adaptation to larger scale processes. Regeneration to a near-peak turbidity was also found to be more efficient under the conditions of the experiment.

An apparatus was assembled having one inoculum and three culture vessels, for operation in a cyclic continuous system. The inoculum vessel was transferred at 5-, 4-, and 3-hour intervals, with respective generation periods for each reactor of 15, 12, and 9 hours. Each schedule was maintained for at least nine transfers, or three complete cycles. Results are given in table 2. These results indicate that for a given schedule, an equilibrium which was rather constant was attained in the system. It appeared that a 4-hour interval between harvests was feasible; a 3-hour schedule apparently resulted in a disproportionally decreased count in the product. Obviously, employment of one inoculum and three culture vessels represented but one of several methods of obtaining maximum utilization of a given number of vessels; in practice, this factor would be adjusted to specifications required for yield and modified to available facilities.

TABLE 1

Regeneration time of aerated (shaker) cultures of Brucella suis

NO.	PARENT CULTURE		SUBCULTURE				
	Light transmittance	Viable cells $\times 10^{-9}$	Amount of inoculum	Regeneration time	Light transmittance	Viable cells $\times 10^{-9}$	Efficiency*
	%	*per ml*	%	*hours*	%	*per ml*	
1	40.0	22	50	5.5	40.0	28	3.1
2	40.0	22	10	10.5	40.0	28	2.5
3	30.5	30	50	7.0	30.0	30	2.1
4	30.5	30	10	11.5	28.5	37	3.0
5	20.5	36	50	10.5	20.0	55	3.5
6	20.5	36	10	16.0	20.0	59	3.5

$$* \text{ Efficiency} = \frac{\text{final count} - \text{initial count}}{\text{regeneration time}} \times 10^{-9}.$$

As mentioned before, the cyclic continuous system may be applied to a unit operation; i.e., a single culture vessel may be used for culture, merely retaining sufficient product after each phase to start the ensuing one. Such a system was examined in some detail, as it afforded a readily adaptable, simple system for larger scale operations. Duplicate sets of apparatus were made for testing

TABLE 2

Operation of multiple cyclic continuous culture system with 50 per cent inoculum*

SERIES NO.	INOCULUM VESSEL			CULTURE VESSELS			
	Age of culture	Light transmittance	Viable cells $\times 10^{-9}$	No.	Age of culture	Light transmittance	Viable cells $\times 10^{-9}$
	hours	%	*per ml*		*hours*	%	*per ml*
1-1	14	38.0		1	15	16.0	56
	5	32.0		2	15	19.5	49
	5	26.0		3	15	19.0	43
1-2	5	28.0		1	15	18.0	47
	5	27.5		2	15	19.0	54
	5	26.0		3	15	20.0	53
1-3	5	26.5		1	15	19.0	50
	5	27.0		2	15	19.5	45
	5	25.0		3	15	17.5	48
1-4	5	26.0		1	14	18.0	51
	5	28.0		2	13	17.5	51
	5	27.0		3	12	17.0	55
1-5	4	31.0		1	12	17.5	46
	4	32.5		2	12	20.0	54
	4	32.0		3	12	16.0	57
1-6	4	31.0	32	1	12	17.0	54
	4	31.0	26	2	12	19.0	53
	4	33.0	31	3	12	19.5	49
1-7	4	35.5	30	1	12	17.5	46
	4	35.0	32	2	12	18.5	35
	4	35.0	31	3	12	17.5	36
1-8	4	36.0	29	1	12	17.0	43
	4	31.0	27	2	12	18.5	46
	4	33.0	21	3	12	19.5	42
1-9	4	32.5	26	1	12	18.5	46
	4	30.0	24	2	12	21.0	26
	4	31.0	20	3	12	19.0	32
2-1	20	30.0		1	9	19.0	36
	3	35.5	15	2	9	31.5	25
	3	35.5	18	3	9	25.5	13
2-2	3	37.5	14	1	9	22.0	32
	3	36.5	15	2	9	22.0	26
	3	41.5	14	3	9	21.0	34
2-3	3	46.0	12	1	9	22.0	32
	3	43.5	11	2	9	22.0	26
	3	41.5	18	3	9	21.5	18
2-4	3	39.5	13	1	9	20.5	22
	3	33.0	15	2	9	20.0	21

* One inoculum vessel and three culture vessels.

10 and 50 per cent inoculum operations. Results of the experiments are given in tables 3 and 4. The efficiency of generation was expressed as increase in cell

TABLE 3

Operation of unit cyclic continuous culture system with 50 per cent inoculum

SERIES NO.	AGE OF CULTURE	LIGHT TRANSMITTANCE		VIABLE CELLS × 10^{-9}		EFFICIENCY*
	hours	*%*	*avg. %*	*per ml*	*avg. per ml*	*avg.*
1	22	18.5	11.7	22	56	2.3
2	12	16.0		40		
3	12	10.5		56		
4	12	13.5		46		
5	12	11.5		60		
6	12	11.0		61		
7	12	12.0		56		
8	8	12.5	12.8	52	50	3.1
9	8	11.0		55		
10	8	15.0		46		
11	8	13.0		45		
12	8	12.0		47		
13	8	13.0		48		
14	8	13.0		54		
15	8	12.5		53		
16	6	16.0	17.4	29	33	2.8
17	6	16.0		23		
18	6	17.0		38		
19	6	18.0		39		
20	6	18.0		30		
21	4	18.0		29		
22	8	18.5		35		
23	6	17.5		40		
24	4	19.5	20.0	19	29	3.6
25	4	22.0		28		
26	4	22.0		27		
27	4	19.5		28		
28	4	19.0		30		
29	4	19.0		32		
30	2	26.0	var.†	19	var.†	var.†
31	2	30.5		17		
32	2	41.5		17		
33	2	44.0		10		
34	2	46.0		14		
35	2	46.5		10		
36	2	48.5		12		
37	2	52.0		8		
38	12	15.0		35		
39	12	12.5		51		
40	12	12.0		56		

$$* \text{ Efficiency} = \frac{\text{final count} - \text{initial count}}{\text{age of culture}} \times 10^{-9}.$$

† Var.: no calculations possible as culture failed to reach equilibrium.

count per hour. It may be seen that higher efficiency figures were obtained for the 50 per cent system; this served to substantiate the preference for a 50 per cent level. Furthermore, the highest efficiency was obtained when transfers were made at 4-hour intervals. Highest counts were, of course, found at the longest interval. In practice, the balance of efficiency vs. cell count per unit volume of medium would be established by the requirements of the product. It was of interest to note that when a 2-hour transfer schedule was maintained, the culture failed to regenerate to the previous level and was merely being diluted out. Thus, it would appear that the generation time of the organism is greater than 2

TABLE 4

Operation of unit cyclic continuous culture system with 10 per cent inoculum

SERIES NO.	AGE OF CULTURE	LIGHT TRANSMITTANCE		VIABLE CELLS × 10^{-9}		EFFICIENCY*
	hours	%	*avg/%*	*per ml*	*avg per ml*	*avg*
1	24	15.0	12.0	47	51	1.9
2	24	12.0		59		
3	24	13.0		53		
4	24	12.0		51		
5	24	12.0		52		
6	24	12.5		48		
7	24	12.0		45		
8	16	12.0	15.5	49	47	2.6
9	16	15.5		45		
10	16	15.0		40		
11	16	15.0		56		
12	16	16.5		42		
13	16	15.5		53		
14	12	20.0	19.5	25	31	2.3
15	12	20.0		24		
16	12	20.0		37		
17	12	18.5		31		
18	8	29.0	31.0	18	18	2.0
19	8	27.0		19		
20	8	36.0		17		
21	10	23.5		20		
22	6	21.0		27		
23†	8	76.0				
24	8	33.0		18		
25	8	31.0		25		
26	8	29.5		22		
27	8	30.5		12		
28	8	31.0		15		
29	8	29.0		17		

* Efficiency $= \frac{\text{final count} - \text{initial count}}{\text{age of cultre}} \times 10^{-9}$.

† Air supply accidentally cut off at this point.

hours; previous calculations from growth curves indicated it to be approximately 2.6 hours. This unit system was operated continuously for as many as 15 days; in every case contamination arose in the medium storage flasks, where it was necessary to open the system infrequently to replenish the medium supply.

As a consequence of these preliminary studies, an apparatus was developed in which the addition of fresh medium and the withdrawal of product were accomplished continuously and simultaneously; its operation has previously been described in detail and a diagram is given in figure 1. Although most of the time available was devoted to designing the apparatus and modifying it for efficiency, a few satisfactory runs were made. The results of one experiment are given in table 5. Samples were taken at 24-hour intervals, in which period it was presumed that the system had reached equilibrium. Although the results are limited, it would appear that an excellent efficiency can be obtained by the process, comparable or better than those from previous cyclic continuous systems. Thus, employing the "optimum" schedule in each case, this completely continuous system gave a harvest of an equivalent full-vessel volume (400 ml) every 7.7 hours with a count of 44 $\times$ 10^9, whereas the best previous system gave an equal amount of product every 8 hours with a count of 29 $\times$ 10^9. Expressed as efficiency (cells per ml increase per hour, $\times$ 10^{-9}), they were 3.6 and 5.7, respectively. The experiment was stopped when contamination appeared in the medium storage vessel, after a replenishment of the medium became necessary. The apparatus was then modified to include a second medium storage carboy (approximately 20 liters), from which medium could be replenished through a completely closed system. With this modification, the apparatus was successfully operated for more than 2 weeks, with a complete turnover in the culture vessel every 8 hours, at most, and with a product having a count in excess of 40 $\times$ 10^9 cells per ml. Preliminary strain studies had shown that strain mutation was minimal after as many as 63 serial transfers, and the same strain constancy apparently occurred in these experiments. No virulence tests were conducted.

TABLE 5

Operation of completely continuous culture system with varying rates of operation

AGE OF CULTURE	CALCULATED RATE OF OPERATION		LIGHT TRANSMITTANCE	VIABLE CELLS $\times 10^{-9}$	EFFICIENCY*
	Replacement per hour	Time for complete replacement			
hours	%	*hours*	%	*per ml*	
24	6.0	16.7	12.5	58	3.5
48	13.0	7.7	22.0	44	5.7
72	14.3	7.0	22.0	25	3.6
96	26.8	3.7	37.0	23	6.2

* Efficiency $= \dfrac{\text{average count}}{\text{time for complete replacement}} \times 10^{-9}$.

DISCUSSION

Although maintenance of bacterial cultures by serial transfers is a routine and accepted procedure, few efforts have been made to study serial transfer in a continuous phase, beyond theoretical considerations. Such a procedure, however, presumably represents the ultimate in efficiency when production of comparatively large quantities of microorganisms or their by-products is desired, and thus assumes practical importance. Application of this principle to the culture of *Brucella suis* resulted in the development of a laboratory apparatus in which the addition of fresh medium and the withdrawal of product were accomplished continuously and simultaneously. It was operated safely and aseptically for prolonged periods of time with little manual attention, and gave a product of very high cell concentration, uniformity, and purity. The advantages of such a continuous process are primarily an increase in production efficiency and reduction in manual attention. With the customary "batch" methods, it had previously been possible to produce a given large unit of *Brucella suis* in approximately 48 hours over-all time; whereas, using a completely continuous system, it is believed that an equivalent product could be produced in approximately 8 hours, not including time which may be gained from the elimination of frequent sterilization of equipment and handling. Once started and adjusted, the continuous system required only infrequent attention to replenish the medium supply and to remove the product. Operated under negative pressure, the system minimized the hazards of producing a highly virulent organism in quantity. The product contained a very high concentration of cells, was free from contamination, and contained practically no morphological strain variants.

The disadvantages of the process are also evident. Strain mutation, particularly from the standpoint of virulence, has not been fully studied and might possibly arise as an objection. More extensive use of the apparatus might introduce increased problems of contamination control. It might be necessary to convert the process to positive pressure, with accompanying difficulties. Foaming of the medium constantly provides a difficult problem of control. These problems will, of course, require further study.

It is believed that, going beyond the scope of this investigation, the process might find successful application to the experimental preparation of vaccines and other immunogenic products, antibiotics, legume inoculants, yeast, and other fermentation products. The fact that the apparatus has been operated with no infections of personnel or serious accidents, in growing an organism as virulent and infective as *Brucella suis*, is considered to be of significance. The utilization of a closed system for culture and the decreased amount of manual attention required have contributed materially to this factor of safety. Thus, the possible applications to experimental preparation of virulent or toxic biologicals are felt to be of particular importance. There are several factors limiting such applications: the organism must have stable strain characteristics; there can be no accumulation of limiting toxic by-products of the organism; and the method probably could not be adapted to the production of sporulating bacteria. With continued study and modification, however, it is hoped that the concept of continuous culture of bacteria may find further practical applications.

SUMMARY

Preliminary experiments were conducted to determine the time required for regeneration of *Brucella suis* cultures, using different levels of inocula taken from various periods in the growth phase. These results were applied to the construction and operation of so-called "cyclic" continuous culture systems, in which the addition of fresh medium and the withdrawal of culture were made at periodic intervals, a portion of the product being retained as starter for the ensuing cycle.

As a consequence of these initial studies, a laboratory scale apparatus was developed in which the addition of fresh medium and the withdrawal of product were accomplished continuously and simultaneously. This apparatus was operated safely and without contamination for prolonged periods of time with little manual attention, and produced a *Brucella suis* suspension of very high concentration, uniformity, and purity.

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A CRITICAL EVALUATION OF THE NITROGEN ASSIMILATION TESTS COMMONLY USED IN THE CLASSIFICATION OF YEASTS

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The ability of some groups of closely related yeasts to use potassium nitrate as a source of nitrogen was applied successfully by Stelling-Dekker (1931) to the classification of the sporogenous yeasts. Later, Lodder (1934) added other nitrogen compounds, namely, ammonium sulfate, urea, asparagine, and peptone, in diagnostic tests for her classification of the nonsporogenous, nonfilamentous yeasts. She employed a modification of Beijerinck's (1889) auxanographic technique in the following manner: About 2 ml of a dense suspension of the yeast to be tested were placed in a petri dish. It was assumed on the basis of Wildiers' (1901) work that the use of such a heavy inoculation of cells would provide adequate growth factors. An agar medium consisting of 2 per cent glucose, 0.1 per cent potassium dihydrogen phosphate, 0.05 per cent magnesium sulfate, and 2.0 per cent washed agar was cooled to 40 C and poured into the dish. The medium and yeast were quickly mixed. After the medium had solidified, the plate was placed in an incubator to dry for a few hours at 30 C. Then small portions of the nitrogen-containing compounds were placed on the solid surface of the inoculated agar. On incubation at 25 C, an area of growth was produced around those compounds that were assimilated.

Lodder's study disclosed that a majority of the yeasts with which she worked were capable of utilizing all the compounds that she had introduced. However, some species of *Torulopsis* and all species of *Kloeckera* failed to assimilate ammonium sulfate, urea, and asparagine. These facts were subsequently incorporated in her descriptions of species and genera.

These nitrogen compounds, as well as the use of the auxanographic plate method, were generally adopted for diagnostic purposes by succeeding workers in Europe and South America. Langeron and Guerra (1938) used Lodder's medium and technique for their study of filamentous yeasts belonging to the genus *Candida*. Because they found that urea diffused through the medium so rapidly that it sometimes overlapped the diffusion zones of other nitrogen sources, only one other test substance was placed in the same plate. Langeron and Guerra found that 2 of their 16 species of *Candida*, *C. pelliculosa* and *C. zeylanoides*, utilized peptone only, but 6 assimilated urea.

At variance with these results were the findings of Diddens and Lodder (1942) with respect to the assimilation reactions of the *Candida* species. They found that *C. pelliculosa* and *C. zeylanoides* utilized ammonium sulfate, urea, and asparagine in addition to peptone, and that the species *C. tropicalis*, *C. guil-*

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liermondii, and *C. zeylanoides* utilized urea, in contradiction of the findings of Langeron and Guerra.

Further confusion was added by the results of Mackinnon and Artagaveytia-Allende (1945). Using Lodder's auxanographic technique, these authors found *C. pelliculosa* able to utilize asparagine and peptone but not ammonium sulfate or urea. They did not study *C. zeylanoides*. Of 15 species of *Candida* studied, only 4 utilized urea. When results with the auxanographic technique were inconclusive, they used Lodder's medium with 1 per cent of the nitrogen compound in tubes, and the growth on the surface was compared with that of a culture on Lodder's medium without added nitrogen source. Zobl (1943) used these nitrogen compounds in a study of the *Candida* species isolated from the vagina. Custers (1940) employed them in his study of the *Brettanomyces* genus.

Yeast taxonomists in North America have not generally used nitrogen or carbon assimilation tests, exceptions being Graham and Hastings (1941), Mrak and McClung (1940), Mrak, Phaff, and Vaughn (1942), Mrak, Phaff, Vaughn, and Hansen (1942), and Bedford (1942). Mrak and Bedford used a synthetic liquid medium rather than auxanographic plates because the liquid method expedited the determinations and gave results comparable to those obtained by the auxanographic method. Mrak and McClung (1940) described a new species, *Torulopsis fermentans*, which assimilated peptone but which did not assimilate asparagine, urea, ammonium sulfate, or nitrate.

EXPERIMENTAL

A study of methods used for assimilation tests was prompted by the need for adequate techniques to be used in the classification of approximately 1,700 yeast strains in the collection of microorganisms maintained by the Northern Regional Research Laboratory. A modification of Lodder's medium was used, in both the solid and liquid form, with additions of trace elements and eight pure B vitamins, seven of which are known to be required by various yeasts.

The medium in current use consists of the following compounds in the amounts indicated per liter of solution:

Trace elements	
Boron, as H_3BO_3	0.01 ppm
Copper, as $CuSO_4 \cdot 5H_2O$	0.01 ppm
Iodine, as KI	0.10 ppm
Iron, as $FeCl_3 \cdot 6H_2O$	0.05 ppm
Zinc, as $ZnSO_4 \cdot 7H_2O$	0.07 ppm
Vitamins	
Biotin	2 μg
Calcium pantothenate	400 μg
Inositol	2,000 μg
Niacin	400 μg
Para-aminobenzoic acid	200 μg
Pyridoxine hydrochloride	400 μg
Thiamine hydrochloride	400 μg
Riboflavin	200 μg

Salts	
KH_2PO_4	0.875 g
K_2HPO_4	0.125 g
$MgSO_4 \cdot 7H_2O$	0.50 g
NaCl	0.10 g
$CaCl_2 \cdot 2H_2O$	0.10 g
Carbon source	
Glucose	10.0 g
Nitrogen sources (used separately)	
$(NH_4)_2SO_4$	1.00 g
KNO_3	0.78 g
Urea	0.46 g
Asparagine	1.00 g
Peptone	1.32 g

The use of stock preparations of trace elements, vitamins, and some of the salts reduce the work required in the preparation of the medium. These stocks are made as follows:

1. Trace elements solution, 1,000 ×. The compounds containing the trace elements are dissolved in water to give a stock solution 1,000 times the concentration desired in the medium. The unsterilized stock is stored in a screw-cap bottle. As mentioned later in this report, passage through a Seitz filter enriches the medium in iron, magnesium, calcium, and probably other trace elements due to the addition of soluble minerals from the filter pad (Browne, 1943; Hunter, 1943; Webb, Irish, and Lyday, 1944). The incorporation of manganese and molybdenum is anticipated in later studies.

2. Vitamin solution, 100 ×. Pure, commercially produced vitamins are suspended in distilled water and pipetted into tubes in such quantities that each tube contains sufficient material for 20 liters of medium. The vitamin suspensions are immediately frozen, dried from the frozen state, sealed under vacuum, and stored in the refrigerator until used. The dried mixture of vitamins from one tube is dissolved in 200 ml of distilled water containing 2.91 g potassium dihydrogen phosphate and 0.06 g potassium monohydrogen phosphate. This 100-fold concentration of vitamins is sterilized by Seitz filtration. The sterile concentrate has a pH of 5.0 to 5.1. It is stored in the refrigerator for use as needed.

3. Salts solution, 10 ×. Ten times the amount of phosphates and sodium chloride required for 1 liter of the medium are dissolved in 900 ml of distilled water. This stock may be kept in a refrigerator without sterilization for a period of 2 or 3 weeks and used as needed.

One-half gram of magnesium sulfate and 0.10 g of calcium chloride are dissolved consecutively in 900 ml of distilled water, and 4.5-ml quantities of the solution are placed in clear test tubes of 16-mm diameter. The tubes are plugged with cotton and sterilized in the autoclave. This procedure prevents the formation of insoluble magnesium ammonium phosphate hexahydrate, which would form were these salts combined in the 10× strength solution.

The stocks are combined in the following manner to prepare 10× strength solutions of each medium to be used. Taking the preparation of the medium with ammonium sulfate as an example, the procedure would be as follows: In 90 ml of the 10× salts solution are dissolved 1 g of ammonium sulfate, 10 g of glucose, and 1 ml of trace elements stock. The solution is sterilized by passage through a Seitz filter. Then 10 ml of the sterile vitamin stock are added. This medium is stored in the refrigerator, and, when needed, 0.5-ml portions are pipetted into the test tubes containing 4.5 ml of the magnesium sulfate and calcium chloride solution prepared as described above. The medium is clear and colorless. The tubes are inoculated usually within 1 or 2 days after they are prepared.

The inoculum is prepared by growing the yeast on slants of yeast extract agar in 16-mm tubes. This medium contains 3 g of powdered yeast extract, 5 g of peptone, 10 g of glucose, and 20 g of agar in 1 liter of distilled water. The pH is 6.8 to 7.0. The yeast is serially transferred twice, and the second slant is used for preparing the inoculum when it is 24 hours old. The procedure consists of pipetting 1 ml of sterile water onto the slant and suspending the growth in it. Then 0.1 ml of the suspension is transferred to a tube containing 3.5 ml of water. After mixing, one drop of the suspension is used to inoculate each tube of medium. Should the inoculum cause a clouding in the medium, it should be diluted further, although this occurs only with yeasts that have grown very abundantly on the slants. The tubes are shaken after inoculation to suspend the cells uniformly; then the cultures are incubated at 28 C and observed for growth at 2 and 4 days.

RESULTS

The use of the medium and techniques outlined above, when applied to species of yeasts reported by various authors to be incapable of utilizing ammonium sulfate, urea, and asparagine, showed that all the strains of these yeasts in our collection which were tested were capable of utilizing these three nitrogen sources. Included were strains representing nine species that have been designated by various authors as incapable of utilizing ammonium sulfate, urea, and asparagine. None of these grew in a control medium consisting of vitamins, trace elements, salts, and glucose, but without any other nitrogen than the extremely minute amount contained in the vitamins.

Figure 1 portrays the growth of these strains in liquid ammonium sulfate medium with and without the addition of vitamins. The same source of inoculum, in equal volumes, was used in each pair of tubes. All of the strains grew in the medium containing vitamins, whereas the three species of *Kloeckera* and two of *Torulopsis* failed to grow in the medium without vitamins.

It was thought that the growth of strains Y-317, *Candida pelliculosa*; Y-109, *C. zeylanoides*; Y-785, *Torulopsis fermentans*; and Y-392, *T. molischiana*, might be due to a transference of sufficient vitamins in the inoculum to overcome the deficiency of the medium. To investigate this possibility the yeasts were transferred serially through the liquid media with and without vitamins. The cultures were transferred at 2-day intervals, using one drop of the shaken culture for inoculation. In the medium without vitamins, Y-1274, Y-915, Y-779, Y-1289, and Y-1293 failed to grow on the first transfer; Y-109 and Y-392 failed to grow on the second transfer; Y-785 failed to grow on the third transfer; but Y-317 grew abundantly on all three transfers. All strains grew throughout all three serial transfers in the medium with vitamins. It is thus apparent that the inoculum of the apiculate yeasts, Y-1274, Y-915, and Y-779, *Torulopsis dattila* Y-1289, and *T. stellata*, Y-1293, did not carry enough vitamins in the original transfer from the agar slant to support growth in the first tube of synthetic medium, whereas the others did. It appears that *Candida pelliculosa*, strain Y-317, is not dependent upon an exogenous supply of vitamins, a finding which agrees with the conclusions of Burkholder *et al.* (1944). The strain used in this study is typical of the species morphologically and physiologically.

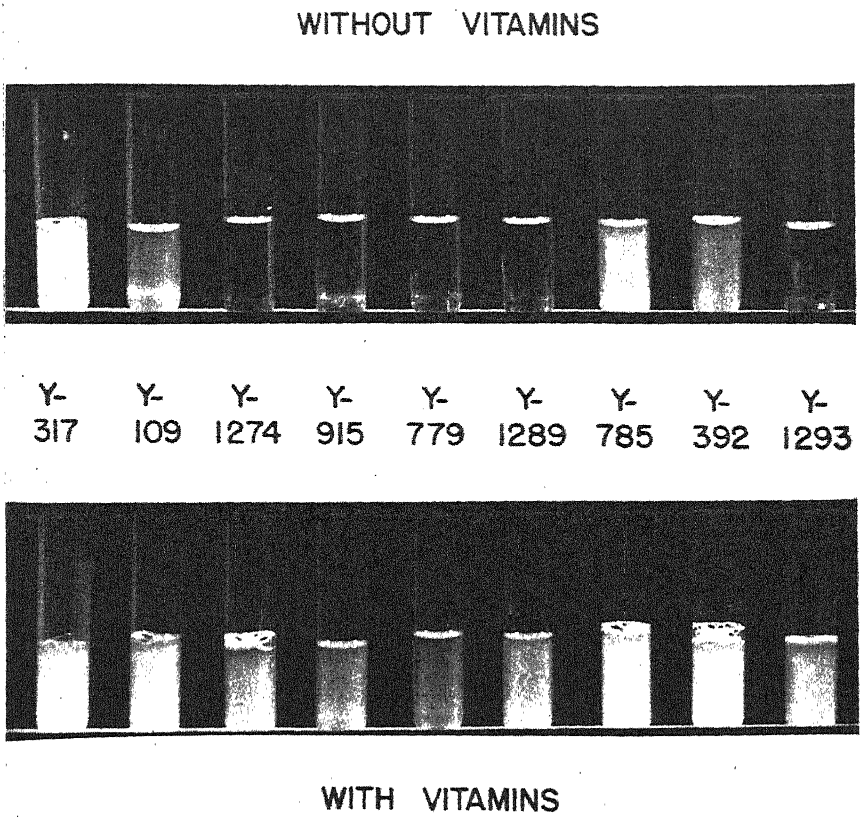

FIG. 1. EFFECT OF VITAMINS ON ASSIMILATION OF AMMONIUM SULFATE BY CERTAIN YEASTS
Liquid cultures, 4 days old

Figure 2 demonstrates that plate cultures of the same media containing 2 per cent agar presented similar results, although the growth in general is less evident than that obtained in the liquid cultures, perhaps because of slow diffusion of the vitamins toward the colonies.

The findings with respect to the utilization of urea obtained by different groups of workers studying the genus *Candida* are interesting because of their diversity. Diddens and Lodder (1942) found the majority of species able to utilize urea; Langeron and Guerra (1938) found the majority of species unable to use urea, and Mackinnon and Artagaveytia-Allende (1945) found only 4 of 15 species of *Candida* able to utilize urea.

Diddens and Lodder stated that the ability of some yeasts to use urea for growth is dependent on the pH of the medium. Since urea in a synthetic medium is toxic at certain pH levels, it would probably follow that at such levels the concentration of the urea would determine whether growth could occur. An experiment proved the validity of this assumption and demonstrated as well that urea may be used as a source of nitrogen by almost all, if not all, yeasts. The species selected for the experiment were those designated by Langeron and Guerra as being incapable of using urea. These authors gave 5.4 as the pH of the medium used in their auxanographic plate technique. This compares with a pH of approximately 5.3 for our liquid medium. We used concentrations of 0.092, 0.046, and 0.023 per cent urea, which are equivalent in nitrogen to 0.2, 0.1, and 0.05 per cent ammonium sulfate, respectively. The results are given in table 1.

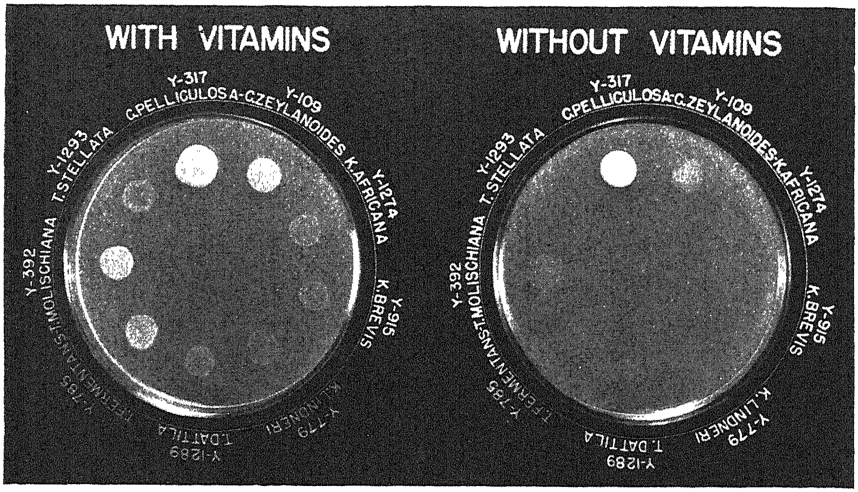

FIG. 2. EFFECT OF VITAMINS ON ASSIMILATION OF AMMONIUM SULFATE BY CERTAIN YEASTS
Solid cultures, 4 days old

Urea in 0.092 per cent concentration inhibited all but 1 of the 10 strains tested, but all grew in the medium containing 0.046 per cent. Why *Candida zeylanoides* in this experiment failed to grow in the medium with 0.023 per cent urea is not known; however, it did grow in this concentration in a subsequent test. The toxicity that results when urea is incorporated into a synthetic medium probably explains why Mackinnon and Artagaveytia-Allende reported only four species of

Candida to use this source of nitrogen, because in all cases in which there was doubt concerning their results with auxanographic plates they used agar slants containing 1 per cent of urea to determine whether growth would occur.

Langeron and Guerra stated that the urea auxanograph had special characters differentiating it from the auxanographs of other nitrogen sources. Their urea auxanographs were late in developing, growth never appearing before 48 hours and generally not until the fifth or sixth day. This may be interpreted as meaning that growth could not occur until the diffusion of urea had reduced its concentration to a nontoxic level.

TABLE 1

The effect of the concentration of urea on its utilization by some species of Candida

STRAIN	SPECIES NAME ACCORDING TO LANGERON AND GUERRA	CONCENTRATION OF UREA		
		0.092%	0.046%	0.023%
Y-980	*Candida aldoi*	−	+	+
Y-311	*Candida brumpti*	−	+	+
Y-982	*Candida chalmersi*	−	+	+
Y-245	*Candida flareri*	+	+	+
Y-324	*Candida guilliermondii*	−	+	+
Y-981	*Candida intermedia*	−	+	+
Y-316	*Candida parakrusei*	−	+	+
Y-317	*Candida pelliculosa*	−	+	+
Y-619	*Candida tropicalis*	−	+	+
Y-106	*Candida zeylanoides*	−	+	−

DISCUSSION

The use of the medium and concentrations of test substances previously outlined, when applied to species of yeasts reported by various authors to be incapable of utilizing ammonium sulfate, urea, and asparagine, showed that all the strains of such yeasts in our collection that were tested were capable of utilizing these three nitrogen sources. The reason these species had been originally designated as not using these compounds was their inability to grow in the vitamin-deficient medium employed by previous workers. The results obtained by Burkholder *et al.* (1944) provide convincing evidence that the apiculate yeasts are dependent to an unusual degree upon the medium for their source of vitamins. In fact, *Kloeckera brevis*, strain Y-915, was found to be deficient for biotin, thiamine, pantothenic acid, nicotinic acid, pyridoxine, and inositol, and was suggested by Burkholder as an assay organism for quantitative estimations of these vitamins. It is of interest to note that the source of nitrogen used by Burkholder was asparagine. Lodder's definition of the genus *Kloeckera* states that asparagine is not utilized by the species of this genus.

In the past the use of ammonium sulfate, urea, asparagine, and peptone assimilation tests has undoubtedly been an aid in the classification of yeasts. With the additional information reported in this paper, these tests now lose their significance as indicating species differences in the utilization of these compounds.

Instead, they indicate species differences in the ability to synthesize certain B vitamins not present in the inadequate medium previously used for these tests.

SUMMARY

The ability of yeasts to utilize ammonium sulfate, urea, asparagine, and peptone has been used for the past 12 years in the classification of yeasts. The present report demonstrates that all the yeasts tested that were previously designated as incapable of assimilating the first three of these compounds will utilize them when an adequate supply of pure vitamins is added.

Urea is toxic when used in simple synthetic media, and its concentration must be low in order to obtain growth with all yeasts.

ACKNOWLEDGMENT

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A NEW MEDIUM FOR THE DETECTION OF UREA-SPLITTING ORGANISMS[1]

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Bacteriological laboratories engaged in diagnostic or research problems concerning pathogenic enteric bacilli, namely, the *Salmonella* and *Shigella* groups, invariably find that members of the genus *Proteus* are by far the most misleading. A dependable, easily interpreted, routine differential test medium for screening the latter group of bacilli becomes desirable. Urease production is a characteristic activity of the genus *Proteus* (Bergey *et al.*, 1939). The hydrolysis of urea by urease has been described by Werner (1923) as "an 'alkaline fermentation' during which the 'carbonate of ammonia' was formed." Rustigian and Stuart (1941) recommended a urea medium for the detection of this enzyme activity. A modification of the latter medium has been described by Anderson (1945). Since one of the products of the splitting of urea is the formation of ammonia, Rustigian and Stuart suggested that phenol red indicator be employed to detect this alkaline change colorimetrically. Howell and Sumner (1934) reported that the pH optimum for urease activity upon 2.5 per cent urea is 6.9 with phosphate buffer.

The present report describes a new medium for the detection of urea-splitting organisms. Colorimetric changes are sharp and permit easy interpretation of results.

METHODS AND MATERIALS

I. The medium is prepared in three parts:

A. The buffered semisolid "deep,"

Bacto tryptose (Difco)	10.0 g
Sodium chloride	5.0 g
Bacto agar (Difco)	3.0 g
Dipotassium phosphate	1.5 g
Monopotassium phosphate	1.0 g
Distilled water	1,000.0 ml

Heat to boiling to dissolve the medium completely. The pH should be 6.9. Add

Meta-cresol sulfon phthalein (0.4 per cent alcoholic solution)	10.0 ml

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[1] This work is a joint project of the Quartermaster Food and Container Institute for the Armed Forces, Chicago, Illinois, and the University of Nebraska, College of Medicine, Omaha, Nebraska.

Tube in 4-ml amounts in 10 by 1.2 cm test tubes. Sterilize in the autoclave for 15 minutes at 15 pounds' pressure. Cool and store in the refrigerator.

B. The "urea" overlaying solution,

Urea	25.0 g
Sodium chloride	5.0 g
Dipotassium phosphate	1.5 g
Monopotassium phosphate	1.0 g
Distilled water	1,000.0 ml

The pH should be 6.9. Add

Meta-cresol sulfon phthalein (9.4 per cent alcoholic solution)	10.0 ml

Sterilize by Seitz (or Berkefeld) filtration. Distribute in approximately 10-ml amounts and store in appropriate sterile screw-cap containers in the refrigerator.

C. The "urea-free" overlaying solution,

Same formula as in B, except for omission of urea. The solution may be sterilized in the autoclave, since urea is not present.

II. The test procedure:

The inoculation is made from a nutrient or Kligler's iron agar slant. A large inoculum on a straight needle is stabbed into the center of each of two tubes of buffered semisolid "deep." The surface of one is covered with 0.2 ml of urea overlaying solution. The second "deep" is overlaid with 0.2 ml of urea-free solution and serves as the control.

III. Interpretation of reactions after 18 to 24 hours' incubation at 37 C.

A. Colorimetric reaction (yellow to purple) diffusing from the surface and beyond half the length of the "deep," ++, positive.

B. Colorimetric reaction diffusing from the surface but *not* beyond half the length of the "deep," +, positive.

C. Colorimetric change at the surface of the "deep," ±, and no change in color, −, negative.

RESULTS AND DISCUSSION

Rustigian and Stuart noted pH changes as high as 9.4 for members of the genus *Proteus* cultivated in their urease test medium. The one exception was *Proteus morganii*, which attained a pH of 8.2 following 48 hours of cultivation.

The medium herein described contains the indicator *meta*-cresolsulfonphthalein, whose deepest color change occurs at pH 8.3. The buffered semisolid "deep" permits optimum growth of organisms with maximum urease production. The urea overlaying solution encourages the maximum urease activity at its optimum pH of 6.9. Positive results are usually discernible after 6 to 8 hours and are very prominent after 18 to 24 hours' incubation 37 C.

Table 1 lists 97 cultures of species of *Proteus*. All gave marked positive (++) reactions in 24 hours. Cultures of paracolon, *Shigella*, *Salmonella*, and *Eberthella typhosa* organisms all gave negative reactions. Two typical cultures of *Pseudomonas aeruginosa*, which readily produce alkalinity and actively proteolyze milk, apparently failed to attack urea. Two cultures of *Alcaligenes faecalis* and three cultures of *Escherichia coli* were urease-negative. Koser (1918) observed that *Aerobacter aerogenes* attacked uric acid as a source of nitrogen. A culture from our stock collection, typical of the species described in Bergey's manual, was examined for urease activity and found to give a definite positive (+) reaction. This culture was also positive in Anderson's modified urea medium. Another interesting observation was made on a culture of *Brucella abortus*.[2] Bergey's manual states that "ammonia is produced from urea" by *Brucella melitensis* and that "the cultural characters [of *Brucella abortus*] are similar to those of *Brucella melitensis*." We noted that *Brucella abortus* attacked urea, giving a definite positive (+) reaction, whereas the control remained negative. This too was confirmed in Anderson's modified urea medium.

TABLE 1

Urease reactions of some gram-negative bacilli

CULTURE	NUMBER	NEW UREASE MEDIUM		UREA-FREE CONTROL	ANDERSON'S MODIFICATION
		6–8 hours	18–24 hours		
Shigella ambigua	2	–	–	–	–
Salmonella types*	19	–	–	–	–(10) doubtful(9)
E. typhosa	3	–	–	–	–
Paracolon species	37	–	–(36) ±(1)	–	–(36) doubtful(1)
Proteus species	74	+	++	–	+
Proteus morganii†	16	+	++	–	+
Proteus morganii (stock collection)	3	+	++	–	+
Proteus vulgaris (stock collection)	4	+	++	–	+
Alcaligenes faecalis	2	–	–	–	–
Pseudomonas aeruginosa	2	–	–	–	–
E. coli v. *communior*	2	–	–	–	–
E. coli v. *communis*	1	–	–	–	–
Aerobacter aerogene	1	–	+	–	+
Brucella abortus	1	–	+	–	+

* *S. pullorum*, 6; *S. anatum*, 2; *S. paratyphi* B, 5; *S. enteritidis*, 4; *S.* species (unidentified), 2.

† Atypical strains of *P. morganii* from an investigation in progress.

In general, correlation of results with Anderson's modified urea medium was particularly good for members of the genus *Proteus*. However, in our hands, a few cultures of *Salmonella* types were difficult to interpret at the end of the half-hour incubation interval recommended by Anderson.

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[2] Culture obtained through the courtesy of the Seventh Service Command Medical Laboratory, Fort Omaha, Nebraska.

The use of a nitrogenous substance, such as bacto tryptose, in the urea medium may be theoretically criticized on the basis of possible ammonia production. However, actively proteolytic and alkaline-producing organisms, such as *Pseudomonas aeruginosa* and *Alcaligenes faecalis*, that have been studied have failed to show any colorimetric changes in the test medium at the end of the 24-hour incubation period. Furthermore, of 167 cultures studied (table 1), a definite positive colorimetric change has not once been noted in the urea-free controls during the specified time interval.

SUMMARY

A new medium for the detection of urea-splitting organisms is described.

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"FALSE POSITIVE" HEMOAGGLUTINATION BY ALLANTOIC FLUIDS OF EMBRYONATED EGGS INOCULATED WITH UNFILTERED THROAT WASHINGS

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The recognition of hemoagglutination in the presence of influenza viruses has greatly simplified virology. Since Hirst's (1941) observation, several other agents such as the viruses of vaccinia (Clark and Nagler, 1943) and mumps (Levens and Enders, 1945) and a pleuropneumonialike organism (Van Herick and Eaton, 1945) have been found to produce this as yet unexplained phenomenon. These reactions can be readily differentiated from one another since they are inhibited by specific antisera. Occasionally in this laboratory certain bacterially contaminated allantoic fluids, obtained from live chick embryos 48 hours after inoculation with unfiltered but penicillin-treated throat washings, have also been found to react with chicken cells. This hemoagglutination may be present even in dilutions of 1:80 and 1:160 and is grossly indistinguishable from that associated with PR-8 or Lee influenza viruses. Unlike those reactions, however, it is inhibited by very small amounts of "normal" rabbit or human sera.

The inoculation of unfiltered throat washings into chick embryos is currently becoming a diagnostic procedure (Rickard *et al.*, 1944; Hirst, 1945; Rose *et al.*, 1945; Salk *et al.*, 1945), and so the existence of this type of "false positive" hemoagglutination must be recognized. It was encountered here four times during the past year.

The reaction is given by fluids which are relatively clear and which come from live embryos. Consequently, differentiation from the amorphous, coarsely granular precipitate of chicken cells sometimes associated with turbid, heavily contaminated fluids (Salk *et al.*, 1945) is possible by inspection. Nevertheless, on each occasion that "false positive" agglutination was encountered, it was associated with bacterial contamination. Three times it was with a mixed flora identified merely as streptococci, staphylococci, *Neisseria*, or gram-positive rods, and once with a pure culture of a hemolytic *Bacillus subtilis*.

The circumstances surrounding this last instance illustrate clearly the need for recognizing this "false positive" agglutination. Throat washings with nutrient broth were obtained on January 2, 1946, from a patient (M) on the fourth day of an influenzal illness later shown by agglutination-inhibition tests (Florman and Crawford, 1944) to have been caused by an influenza type B virus. The washings were stored at approximately −20 C until January 7. On that day they were slowly thawed, sufficient penicillin was added to yield a concentration of 1,000 units per ml, and each of six 11-day-old embryonated eggs was inoculated with 0.1 ml by the allantoic route. Four embryos survived 48 hours' incubation at 37 C. None of their allantoic fluids agglutinated chicken cells. A second passage was therefore made with the pooled fluids. This time all the embryos survived 48 hours at 37 C and, after an additional 18 hours at 4 C, the allantoic fluid from one gave a characteristic "influenzalike" agglutination (Florman and Crawford, 1944). Since the patient had already been shown serologically to have had a type B influenza, it was thought that the causative agent had been isolated and the fluids were stored in the refrigerator over the week end pending confirmatory studies. Approximately 48 hours later all the fluids were retested, and this time, in addition to the one positive previously noted, three others also gave the characteristic hemoagglutination pattern.

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On January 15 a third egg passage was made. Two pools were prepared each containing two of the agglutinating fluids. Then 1:10, 1:100, and 1:1,000 dilutions in saline were made and inoculated into a total of 36 embryos, all of which survived 48 hours' incubation at 37 C. However, only the pool containing the fluid which had originally agglutinated the chicken cells yielded any hemoagglutinating allantoic fluids. Of the 18 embryonated eggs which had received this pool, 7 gave positive reactions. Three were obtained from the six embryos which had been given the inoculum diluted 1:1,000, three from the six given the inoculum diluted 1:100, and only one from those given the 1:10 dilution. All of these positive fluids were pooled. The hemoagglutinating titer of this pool was found to be 1:80. An agglutination-inhibition test was then set up with two high-titered rabbit sera, one for type A and the other for type B influenza. Agglutination was markedly inhibited by both sera. Therefore the agglutination-inhibition experiment outlined in table 1 was performed the following day. The results clearly demonstrate the nonspecific nature of the agglutination reaction.

TABLE 1

Inhibition of "false positive" hemoagglutination by various sera

SERA	SERUM DILUTIONS				SALINE CONTROL
	1:10	1:50	1:250	1:1,250	
Normal rabbit	0	0	0	1+	4+
Influenza A(PR-8) rabbit	0	0	0	4+	
Influenza B(Lee) rabbit	0	0	0	4+	
Normal human (low A & B)	0	0	0	0	
Influenza A human (high A, low B)	0	0	0	0	
Influenza B human (high B, low A)	0	0	0	±	
Patient (M)—acute	0	0	0	0	
Patient (M)—convalescent	0	0	0	0	

4+ = agglutination. Well-defined pattern of sedimented cells which does not run when tube is tilted. 0 = no agglutination.

An unsuccessful attempt was made to determine whether the reaction could have been caused by the *B. subtilis* recovered from the pooled fluids used in the agglutination-inhibition test. Smears of the agglutinated cells stained with Wright's technique revealed relatively few bacteria and no apparent association between the clumps of chicken cells and the bacteria. A fourth passage of this pooled fluid diluted 1:100 was made to eight eggs. Although after 48 hours all were contaminated with *B. subtilis*, only five produced hemoagglutination. These, however, were the fluids which had the greatest contamination. The organisms from an overnight growth of this strain of *B. subtilis* in tryptose phosphate broth after storage at 4 C for 24 hours were precipitated by centrifugation, washed once with saline, and used to prepare a slightly turbid suspension in normal allantoic fluid. The supernatant from this culture was passed through a Mandler no. 6 filter. Both the bacterial suspension and the bacteria-free filtrate failed to produce any agglutination when tested against chicken cells.

The substance in these allantoic fluids which is responsible for this hemoagglutination must be somewhat labile. Although a titer of 1:80 could be obtained with the fluid from the third passage after 2 days' storage in a plugged Erlenmeyer flask at 4 C, no agglutination at all could be demonstrated after 4 days at that temperature. This is in striking contrast to allantoic fluids containing the PR-8 and Lee influenza viruses, which even at room temperature maintain satisfactory hemoagglutinating titers for at least as long as 61 days (Florman and Weiss, 1945).[2]

SUMMARY

After inoculation of chick embryos with unfiltered penicillin-treated throat washings, certain bacterially contaminated allantoic fluids, even in relatively high dilutions, produce agglutination of chicken cells grossly indistinguishable from that caused by influenza viruses. This reaction, which has been called "false positive," is inhibited by very small amounts of "normal" rabbit and human sera. It is thus readily differentiated from hemoagglutination in the presence of viral agents. Although associated with bacterial contamination, the phenomenon could not be reproduced by the bacteria alone or by a bacteria-free filtrate of a culture. The causative agent is unknown.

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[2] Burnet and Stone (Burnet, F. M., and Stone, J. D., Haemagglutinins of vaccinia and ectromelia viruses, *Australian J. Exptl. Biol. Med. Sci.*, *24*, 1–8, 1946) have recently reported that certain as yet unidentified tissue lipids of chick and animal origin may agglutinate red cells of some species. This nonspecific lipid agglutination resembles the phenomenon reported here in that it is also inhibited by normal serum in high dilution.

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DECOMPOSITION OF TARTRATES BY THE COLIFORM BACTERIA

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Tartrate decomposition has been reported at least since 1841 when Nöllner first published on the end products of calcium tartrate decomposition. The chemists in the period 1841 to 1858 were not aware of the microbial cause of the decomposition of the tartrates whose end products they analyzed. Even the influence of Pasteur, who first recognized and recorded microbial decomposition of tartrates (1858) and decomposition by the mold *Penicillium glaucum* (1860) and by anaerobic bacteria (1863), was not enough to overcome the tendency of analysts to ignore the microbiological problems and content themselves with the study of the spontaneous decomposition of tartrates as late as 1911.

Even today, the majority of the bacteria responsible for the various types of tartrate decomposition are not well known. Only two groups of bacteria whose taxonomic positions are known have been studied in some detail with respect to their ability to decompose tartrates: the coliform bacteria and certain of the lactic acid bacteria found in wines. Although decomposition of tartrates was used by Pasteur for the first proof of anaerobiosis, the anaerobic sporeforming bacteria causing the decomposition were not studied in pure culture until recently and must yet be completely classified (Vaughn and Marsh, 1943*a*, 1943*b*).

The first conclusive evidence of tartrate decomposition by pure cultures of coliform bacteria was furnished by Grimbert and Ficquet (1897, 1898) and Grimbert (1899). The organism used by Grimbert and Ficquet was named *Bacillus tartricus*, which, fortunately, was described in enough detail to be recognizable as a species of *Aerobacter*. Nijdam (1907) accumulated further supporting evidence with his study of tartrate decomposition by a bacterium of the coliform group which he named *Aerobacter tartarivorum*. More recently Barker (1936) and Sakaguchi and Tada (1940) have confirmed these earlier observations by experiments on tartrate decomposition by two different species. Barker worked with *Aerobacter aerogenes*. Sakaguchi and Tada named their bacterium *Bacterium succinicum*, but it is recognizable as a species of *Escherichia*.

Completely irrefutable evidence for tartrate decomposition by *Escherichia coli* and species of the *Enterobacteriaceae* other than those mentioned above is lacking, although many qualitative experiments have been reported, and this qualitative evidence points to the fact that many of these bacteria probably do have the ability to attack tartrates.

The recovery of tartrates, particularly calcium tartrate, as a major by-product of the wine industry in California has developed only recently. Before the war tartrate recovery was incidental and, with almost no recovery of the neutral calcium tartrate, little attention was paid to losses by microbial decomposition.

With the marked increase in tartrate recovery because of the war, losses caused by bacteria and molds became acute. The general aspects of the spoilage problem were discussed by Vaughn and Marsh (1943*b*), who pointed out that the coliform bacteria, particularly *Aerobacter aerogenes*, caused most of the losses.

It was the purpose of the present investigation to determine the extent to which strains of the various recognized species of the coliform bacteria may be involved in tartrate decomposition, to study some of the factors which influence the decomposition of tartrates by coliform bacteria, and to evaluate the possible use of tartrate decomposition as a characteristic for differentiation of the species of these bacteria.

SOURCE OF CULTURES

The cultures isolated were obtained by direct plating on Levine's eosine methylene blue agar. Samples from which the cultures were isolated included crude calcium tartrate of good quality, calcium tartrate of inferior quality suspected of incipient deterioration, spoiled calcium tartrate, tartrate recovery liquors, tartrate wash liquors and water supplies used in the recovery process, residue sludge from the recovery process, soil impregnated with spoiled calcium tartrate, and grape pomace. A total of 26 different isolates were recovered from these sources.

In addition 79 cultures of *Escherichia* and 100 cultures of *Aerobacter* were used as test cultures for some of the studies. These cultures, from the Division of Food Technology collection, originally were isolated from a wide variety of sources including various waters and soils, human and animal feces, olives, chicken eggs, milk, sawdust, oysters, dehydrated and frozen vegetables, food processing equipment, air, etc.

METHODS

All cultures, regardless of source, were subjected to serial replating on Levine's eosine methylene blue agar for purification. Well-isolated colonies were picked, and purified by repeated plating from lactose broth (Am. Pub. Health Assoc., 1936).

Generic differentiation as well as specific allocation of the cultures was made according to the methods suggested by Levine (1921), Levine *et al.* (1934), and Vaughn and Levine (1942). In the taxonomic study all tartrate cultures were incubated at 30 C because of the increasing evidence that 30 C is a more nearly optimum incubation temperature for the coliform bacteria than 37 C, which is generally specified.

Media for study of tartrate decomposition. Tartrates chosen for investigation included ammonium tartrate ($(NH_4)_2\ C_4H_4O_6$), barium tartrate ($BaC_4H_4O_6$), calcium tartrate ($CaC_4H_4O_6 \cdot 4H_20$), copper tartrate ($CuC_4H_4O_6 \cdot 3H_2O$), magnesium tartrate ($MgC_4H_4O_6 \cdot 4H_2O$), sodium tartrate ($Na_2C_4H_4O_6 \cdot 2H_2O$), potassium tartrate ($K_2C_4H_4O_6 \cdot \frac{1}{2}\ H_2O$), sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$), and potassium acid tartrate ($KHC_4H_4O_6$). All were made from dextrorotatory tartaric acid ($C_4H_6O_6$). These salts were used in concentrations of approximately 1 per cent (10 g per liter) in a medium which also contained 5 g of bacto tryptone and 1 g of di-basic potassium phosphate ($K_2HPO_4 \cdot 3H_2O$) per liter. When a solid medium was desirable 15 g of agar were added per liter. For the sake of convenience and accurate duplication, the calcium tartrate medium was prepared by mixing 2 volumes of a double strength basal solution (10 g bacto tryptone and 2 g $K_2HPO_4 \cdot 3H_2O$ per liter) with 1 volume of 0.154 M potassium tartrate solution and 1 volume of 0.154 M calcium chloride solution to give a finished medium containing 1 per cent calcium tartrate.

Later, when the insoluble calcium tartrate made rate studies difficult, an ammonium tartrate medium was used. This medium was prepared by mixing equal portions of 0.135 M ammonium tartrate solution and the double strength tryptone basal medium already described. The resulting medium contained more than 1 per cent of the salt, but the tartrate-ion concentration was about 1 per cent. The method of using a fixed concentration of tartrate ion was considered more advantageous for use with the colorimetric method employed for analysis.

For study of the effect of hydrogen-ion concentration (pH) on the decomposition of tartrates, the media were prepared by mixing ammonium tartrate and tartaric acid solutions with the basal double strength tryptone solution. One volume of the basal medium was mixed with one volume of a mixture of 0.135 M ammonium tartrate and 0.13 M tartaric acid solutions which previous testing showed would give the desired reaction after sterilization. The percentage of each tartrate component for any given pH value was taken from curves prepared for this purpose. These volumetric methods for preparing tartrate-containing media not only reduced errors arising from weighing small individual samples of the tartrate salts but also saved appreciable time. The media were sterilized at 15 pounds' steam pressure for 20 minutes unless the pH value was less than 5.0, in which case 15 minutes was considered sufficient time.

Quantitative determination of tartrate. The procedure used for tartrate determination is based on a modification of the method described by Underhill *et al.* (1931) as modified to photoelectric colorimetry by Matchett *et al.* (1944).[1] The method makes use of the characteristic color developed through the interaction of sodium metavanadate and the tartrate ion in dilute aqueous acetic acid solution.

Before the determination of tartrate content, cloudy or colored samples require clarification and decolorization. In both operations, particularly the latter, tartrate may be lost by absorption and adsorption. Matchett *et al.* prevented this by using an extremely acid reaction. The filtered and decolorized solution was then neutralized before testing. Nearly all activated carbons work satisfactorily under the conditions employed by Matchett *et al.*, but the method becomes highly empirical. This requires that the unknown be determined by steps identical to the standard curve or equation in all respects. Use of a carbon which adsorbs tartrate, even in small amounts, requires careful weight or volume measurement of this material for purposes of duplication. Therefore, it was considered preferable to use a carbon without this property if such existed or could be prepared.

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[1] Also see Western Regional Research Laboratory (1943).

Extensive testing proved that carbon could be prepared with its tartrate-adsorbing property fully satisfied. Treatment of the carbon by the usual acid washing procedure with the addition of 5 g of tartaric acid per liter of acid solution used accomplished this goal. The carbon must be dried before use. Drying is an essential step, but what it actually accomplishes is not known.

Only a single calibration curve need be established for all tartrate-containing compounds provided such a curve is based upon the tartrate-ion concentration. Multiplication of the tartrate-ion concentration obtained by analysis by the appropriate factor converts it to that of the specific compound being determined. The tartrate-vanadate color complex obeys Beer's law when the concentration of tartrate ion in the solution under immediate testing varies from 0.001 to 0.02 per cent, using a 520 filter, and from 0.004 to 0.032 per cent, using a 540 filter in an Evelyn photoelectric colorimeter. The formula for the standard curve with the 540 filter is $\text{Log G} = 0.76 \times X + 2.021$, in which X equals mg of tartrate ion in the 25 ml of reaction mixture.

In the tartrate determination, the preliminary treatment employed depended upon the tartrate media used and the factors under investigation. In all cases except rate studies, the entire contents (100 ml) of the culture flask were transferred to a 250-ml volumetric flask and made to volume with distilled water. With calcium tartrate media, exactly 1.5 ml of concentrated HCl were added to effect solution of the insoluble residue before making this transfer. In the case of rate studies conducted with soluble tartrate salts, a portion of the medium was withdrawn aseptically by pipette from the culture flask. From 10 to 50 ml of solution, depending upon the amount of decomposition judged to have occurred, were taken for determination. After being adjusted to approximately 50 ml with water, the solution was decolorized by boiling for 2 to 3 minutes with about one-quarter teaspoon of the acid-treated activated carbon, allowed to cool, and transferred to a 100-ml volumetric flask. After being adjusted to volume, a portion of the solution was filtered through no. 2 Whatman paper into clean, dry beakers and from 2 to 20 ml were used for colorimetric estimation. Care was taken to remove any bacterial turbidity during this step.

The colorimetric estimation was carried out as follows: The estimated quantity of the foregoing solution was transferred by pipette to 25-ml glass-stoppered mixing cylinders. Distilled water was added to a volume of 20 ml. Then 0.5 ml of glacial acetic acid, 2 ml of a 5.0 per cent sodium metavanadate ($NaVO_3$) solution, and enough distilled water were added to adjust the final volume to 25 ml. After thorough mixing the sample was set aside in subdued light for 30 minutes to allow for color development. The transmittancy of the reaction mixture was then determined by an Evelyn photocolorimeter using the 540 filter. From the galvanometer reading thus obtained and the formula for the standardization curve the residual tartrate content of the original medium was readily computed.

DIFFERENTIATION OF THE BACTERIA

The 26 cultures isolated from the tartrate samples were typical coliform bacteria in that they all produced acid and gas from lactose; were gram-negative, short rods; did not form endospores; and, if motile, possessed peritrichous flagella. Generic differentiation of these cultures was made on the basis of the Voges-Proskauer reaction and Koser's citrate test. Specific allocation was accomplished by investigating the decomposition of sucrose, starch, aesculin, salicin, and glycerol, and the production of hydrogen sulfide and indole in suitable media. The differential characteristics of the bacteria together with their suggested taxonomic allocations are shown in table 1.

TABLE 1

Characteristics of coliform bacteria isolated from tartrates and tartrate-containing materials

NUMBER OF CULTURES*	GENERIC CHARACTERS		SPECIFIC CHARACTERS							SUGGESTED ALLOCATION
			Decomposition of:							
	V.-P. test	Growth in Koser's citrate	Sucrose	Starch	Aesculin	Salicin	Glycerol	H_2S	Indole	
	Number of cultures showing positive reactions†									
10	10	10	10	10	10	10	10	0	2	*Aerobacter aerogenes*
5	0	0	0	3	5	5	5	0	5	*Escherichia coli*
2	0	0	2	2	2	2	2	0	2	*Escherichia neapolitana*
1	0	0	1	1	0	0	1	0	1	*Escherichia communior*
1	0	0	0	1	0	0	1	0	1	*Escherichia acidilactici*
4	0	4	1	0	1	2	4	4	2	*Escherichia freundii*
3	0	3	3	3	3	3	3	0	0	*Escherichia intermedium*

* All the cultures decomposed calcium tartrate in amounts ranging from 25 to 100 per cent of that contained in the media after 7 days at 37 C.

† Based on incubation at 30 C for a maximum period of 4 days.

It is particularly interesting that with the exception of *Aerobacter cloacae* all widely recognized species of *Aerobacter* and *Escherichia* were isolated from the tartrate-containing samples. The most abundant single species was *Aerobacter aerogenes* (10 cultures). It will be noted, however, that the total number belonging to the genus *Escherichia* was greater (16 cultures). The isolation of so many different species from so few samples may perhaps be considered to be fortuitous. Nonetheless, in view of some of the following experimental evidence it is reasonable to believe that representatives of all the species of the coliform bacteria may be involved in tartrate decomposition rather than chiefly species of *Aerobacter* as inferred in the literature already cited; for, in several instances, cultures of *Escherichia* and *Aerobacter* were isolated from the same sample and in others only cultures of *Escherichia* were recovered.

ABILITY OF COLIFORM BACTERIA TO DECOMPOSE TARTRATES

The observation that species of *Escherichia* as well as *Aerobacter aerogenes* were involved in the decomposition of tartrates prompted an investigation of the incidence of tartrate decomposition among a larger collection of coliform bacteria as well as other studies on the decomposition of tartrates.

Incidence of the ability to decompose calcium tartrate. Pure cultures of *Escherichia* and *Aerobacter* from the laboratory collection were used to ascertain the ability of the various species of coliform bacteria to decompose calcium tartrate in the medium already described.

TABLE 2

*The decomposition of calcium tartrate by coliform bacteria**

Genus	ESCHERICHIA						AEROBACTER	
Species	*coli*	*communior*	*acidilactici*	*neapolitana*	*freundii*	*intermedium*	*aerogenes*	*cloacae*
Number	13	8	9	8	24	17	50	50
DECOMPOSITION OF CALCIUM TARTRATE	Number of cultures and range of decomposition							
per cent								
0–9.9†	2	0	0	3	6	9	27	46
10.0–19.9	0	0	4	1	0	0	1	1
20.0–39.9	6	5	1	4	6	5	3	2
40.0–59.9	5	3	4	0	11	3	1	1
60.0–79.9	0	0	0	0	0	0	0	0
80.0–99.9	0	0	0	0	1	0	18	0

* Incubated at 30 C for 7 days.

† None of these cultures attacked the tartrate.

A total of 179 cultures including 79 strains of *Escherichia* and 100 strains of *Aerobacter* were tested. Inoculations were made from 24-hour nutrient agar slant cultures using one loopful of inoculum. The inoculated medium was incubated at 30 C for 7 days before the amount of calcium tartrate decomposition was determined. The results of this investigation are shown in table 2.

These data show that representatives of all species of *Escherichia* as well as *Aerobacter* possess the ability to decompose calcium tartrate. The general lack of ability of cultures of *Aerobacter cloacae* used in this experiment to decompose calcium tartrate may explain why cultures of this species were not recovered from the spoiled tartrates.

Decomposition of various tartrate salts. Several tartrate salts as well as the insoluble calcium tartrate have been used by others who have studied tartrate decomposition by bacteria. It was thought advisable to determine whether the kind of tartrate salt might influence the activity of the coliform bacteria.

The *d*-tartrate salts listed previously were used under the conditions prescribed. All were soluble in 1 per cent concentration (10 g per liter) in the medium with the exception of potassium acid tartrate which was used in a concentration of 0.5 per cent. The species of *Escherichia* and *Aerobacter* isolated from the tartrate-containing materials were used as test cultures. Copper tartrate and tartaric acid were not attacked. Potassium acid tartrate was decomposed by some of the more acid-tolerant cultures. The pH of the medium was low in the case of the tartaric acid (pH 2.6) and potassium acid tartrate (pH 4.1). These low pH values retard or entirely prevent the growth of most coliform bacteria. The amount of copper contained in the copper tartrate medium apparently was responsible for the failure of the test organisms to attack this salt.

TABLE 3

Decomposition of isomeric forms of ammonium tartrate by coliform bacteria

ORGANISM	ISOMERIC FORM OF AMMONIUM TARTRATE*					
	d		*dl*		*l*	
	Percentage of decomposition at 30 C after					
	1 week	2 weeks	1 week	2 weeks	1 week	2 weeks
E. coli	62.9	94.5	61.6	68.2	35.0	82.7
E. communior	66.0	93.3	66.9	69.4	30.0	40.8
E. acidilactici	43.0	91.9	52.1	52.1	24.6	52.3
E. neapolitana	39.3	88.3	20.5	62.5	19.8	59.6
E. freundii	61.7	92.2	45.4	54.7	10.2	32.6
E. intermedium	92.1	94.1	48.6	67.2	29.2	56.5
A. aerogenes	92.8	93.3	86.4	94.1	89.6	95.3
A. cloacae	68.1	96.1	52.1	63.8	14.6	30.4

* Contained in the basal tryptone medium in concentrations of approximately 1 per cent.

All the other neutral *d*-tartrate salts were decomposed by all the cultures tested. Since they were attacked at approximately the same rate and to the same extent, it was decided to use ammonium *d*-tartrate for the studies in which a soluble salt was essential. Other investigators previously had used the ammonium salt. Furthermore, circumstances prevailing at the initiation of these studies dictated the choice. Supplies of chemically pure neutral salts were low, and ammonium *d*-tartrate could be prepared in the laboratory with ease.

The tartrate isomers investigated included the ammonium salts of *dl*(racemic) and *l*(levo) tartaric acid. These salts were used in the basal tryptone medium in concentrations of approximately 1 per cent. Test cultures included the seven species of *Escherichia* and *Aerobacter* commonly recognized. Tartrate decomposition was determined quantitatively after 1 and 2 weeks of incubation at 30 C. The results of this experiment are shown in table 3.

Although all the test cultures could decompose the three tartrate isomers, *Aerobacter aerogenes* was the most active species tested. It also will be noted that the dextrorotatory form in general was attacked more vigorously by all test organisms than either the inactive or levorotatory isomers, even after 2 weeks of incubation, indicating a distinct preference for the *d* form.

Meso-tartaric acid was not investigated. This compound does not occur naturally. Furthermore, the sodium metavanadate method is of no value for determination of *meso*-tartrate.

SOME FACTORS AFFECTING THE DECOMPOSITION OF D-TARTRATE SALTS

The factors showing the greatest influence on the decomposition of *d*-tartrate salts were the temperature of incubation, the reaction (pH) of the medium, and the length of the incubation period.

TABLE 4

The effect of temperature of incubation on calcium tartrate decomposition by coliform bacteria

SPECIES	DAYS OF INCUBATION	TEMPERATURE*							
		16 C	19 C	25 C	28 C	30 C	34 C	37 C	43.5 C
		Calcium tartrate decomposition, per cent							
A. aerogenes (CPGI)†	2	71.6	75.8	62.6	84.7	73.3	71.5	84.2	20.0
A. aerogenes (CPGI)†	2		65.4	76.9	83.2	75.4	82.0	92.6	50.0
A. aerogenes (CPGI)†	7	88.5	93.2	94.2	95.9	95.6	95.3	97.2	94.7
A. cloacae (202/2)	7					51.0	46.9	69.3	38.2
E. coli (JVR)	7					36.2	49.5	30.0	72.9
E. acidilactici (CPA)	7					50.1	53.6	60.7	74.5
E. neapolitana (GW-2)	7					50.1	34.6	58.2	66.3
E. freundii (56/5)	7					45.9	98.8	68.5	19.3
E. intermedium (326)	7					53.6	48.5	51.0	24.0

Blank spaces indicate no culture was grown.

* These temperatures refer to the air temperatures of the various incubators.

† Separate experiments repeated at widely separated intervals.

The effect of temperature. Although the influence of temperature on the growth of coliform bacteria is well known, little attention has been paid to its effect on the ability of the coliform bacteria to decompose various substrates. To determine the effect of the temperature of incubation on calcium *d*-tartrate decomposition by the coliform bacteria representative cultures were grown in the calcium *d*-tartrate medium at temperatures (incubator air) ranging from 16 to 43.5 C. Inoculations were made from 18-hour broth cultures of the organisms using 0.5 ml of inoculum per 100 ml of medium. The inoculated flasks were incubated for 7 days at the desired temperatures and then analyzed for tartrate content. The results of this study are shown in table 4.

The culture of *Aerobacter aerogenes* was active over a wide range of temperatures (19 to 43.5 C), whereas the other test cultures tended to exhibit a definite optimum in a narrower range (30 to 43.5 C). It is interesting in particular that the culture of *Aerobacter cloacae* had an optimum temperature of

37 C for calcium *d*-tartrate decomposition, for this particular strain (202/2) was known to decompose lactose and other sugars very poorly at 37 C. It is also noteworthy that three citrate-negative species of *Escherichia* had higher optima (43.5 C) for calcium *d*-tartrate decomposition than the citrate-positive species (30 to 37 C).

The effect of pH of the medium. Preliminary observations indicated that some of the coliform bacteria associated with tartrate spoilage were active at rather low pH values. To test the effect of pH on the ability of the coliform bacteria to grow and decompose *d*-tartrate, liquid media were adjusted to various pH values between 3.8 and 7.3 as previously described. Inoculations were made from 18-hour broth cultures using 0.5 ml per 100 ml of tartrate medium contained in 300-ml Erlenmeyer flasks. The inoculated media were incubated at 37 C for varying lengths of time, but in most cases for 7 days before analysis. The amount of decomposition occurring is shown in table 5.

TABLE 5

The effect of pH on tartrate decomposition by coliform bacteria

SPECIES	DAYS INCUBATION	pH VALUE*									
		3.8	4.0	4.25	4.5	4.75	5.0	5.35	6.15	6.4	7.3
		Tartrate decomposition, per cent									
A. aerogenes (CPGI)†	1½	0	0	18.7	96.2	95.7	95.7	95.4	96.1	95.9	96.2
A. aerogenes (CPGI)	7	0	8.1	93.8	93.8	93.8	94.4	94.9	95.5	96.0	95.6
A. aerogenes (CAT)	7	0	0	2.8	94.5	91.6	94.2	94.8	95.3	96.1	94.7
A. cloacae (202/2)	7	0	0	0	0	77.7	61.2	62.5	60.0	57.2	45.6
E. coli (JVR)	7	0	0	13.5	89.4	91.6	91.6	83.6	78.1	65.2	54.8
E. coli (JVR)	30	0	0	93.2	93.6	93.7	91.9	90.8	80.3	70.5	58.2
E. communior (WD-1)	7	0	0	0	0	60.4	70.4	71.6	70.8	69.7	68.3
E. acidilactici (CPA)	7	0	0	27.2	94.4	91.4	94.2	94.6	94.5	95.0	94.7
E. neapolitana (GW-2)	7	0	0	0	76.6	64.6	48.5	45.3	43.0	36.7	27.8
E. freundii (56/5)	7	0	0	4.7	5.8	93.6	94.0	95.0	82.3	76.7	61.0
E. freundii (B1)	7	0	0	8.0	96.1	90.4	90.9	92.1	91.6	92.4	92.1
E. intermedium (326)	7	0	0	0	13.1	93.5	93.2	90.0	82.8	77.1	68.8

* pH value within ±0.02 after sterilization.

† This strain of *A. aerogenes* decomposed tartrate within 7 days in media with initial pH values of 3.90.

It will be seen (table 5) that the pH of the medium has a marked effect on the ability of the coliform bacteria to decompose *d*-tartrate. Some cultures exhibit a definite optimum pH value for maximum decomposition. Other cultures show comparable activity over a wide range. The most active culture (*A. aerogenes* CPGI) decomposed the tartrate equally well between pH 4.25 and 7.3 after 1 week of incubation although with shorter incubation a retarding effect was noted at pH 4.25. Some of the less active strains exhibited a definite optimum pH value in the range 4.5 to 5.0. Others showed similar activity between pH 4.5 and 7.3. The difference in activity does not appear to be specific, as is illustrated by the two strains of *Escherichia freundii*.

The pH value which prevented growth was somewhat lower than that which prevented decomposition of the tartrate when cultures were incubated at 37 C for 1 week. This was illustrated by growing 5 cultures of each species of *Escherichia* and 15 cultures of each species of *Aerobacter* in the tryptone tartrate broth adjusted to pH values ranging between 3.92 and 4.50. The medium was contained in 6 by 3/4 inch tubes in 10-ml amounts. The inoculum consisted of one loopful (4 mm diam.) of 18-hour nutrient broth cultures.

The cultures of *Aerobacter aerogenes* tolerated a lower pH than any of the other species tested. Three strains grew at pH 3.92 within 7 days.[2] (These 3 cultures can decompose tartrate at this pH value within 7 days if the inoculum contains bacteria to ensure an initial population of 1 to 3 $\times$ 10^6 organisms per ml of medium.) At pH 4.0 there were 10 cultures which grew. Twelve cultures grew at pH 4.12 within 7 days. At pH 4.24 all 15 strains of *Aerobacter aerogenes* grew luxuriantly within 2 days. In comparison, only one strain of *Aerobacter cloacae* grew at pH 4.12. At pH 4.24, 12 of the strains grew; at pH 4.36, 14 of the 15 *Aerobacter cloacae* strains grew; yet all the cultures did not grow until the pH had been increased to pH 4.50.

The species of *Escherichia* exhibited a marked degree of strain variation. None grew in a medium adjusted to pH 4.12. Some cultures of *Escherichia coli* grew in a medium adjusted to pH 4.24. However, until the reaction was adjusted to pH 4.50 a marked degree of inhibition was noted. Even at pH 4.5 some inhibition was observed as 2 strains of *Escherichia communior* and 1 strain of *Escherichia neapolitana* did not grow.

Rate of decomposition of d-tartrate by coliform bacteria. As indicated by some of the data presented in tables 2 and 4, there are two distinct types of coliform bacteria insofar as destruction of *d*-tartrate is concerned. One group, represented by 21 very active strains of *Aerobacter aerogenes*, decomposes the *d*-tartrate rapidly. The other group, represented by all of the other strains of coliform bacteria investigated, decomposes the *d*-tartrate slowly.

A comparison of the rates of ammonium *d*-tartrate decomposition by these two groups was made. Cultures of *Aerobacter aerogenes* (strain CPGI, rapid group) and *Escherichia coli* (strain JVR, slow group) were used. These cultures were grown in ammonium *d*-tartrate broth prepared to give pH values of 4.5, 5.0, and 7.3 after sterilization. The inoculum consisted of 10 loops (4 mm diam.) of a 24-hour calcium tartrate agar slant culture, suspended in 5 ml of lactose broth, per 1,500 ml of medium contained in a 2-liter Erlenmeyer flask. Samples were withdrawn at desired intervals during incubation at 37 C in order to determine the amount of decomposition. Curves which illustrate the marked difference in rate of destruction of ammonium *d*-tartrate by the two groups, grown in the medium with an initial pH value of 7.3, are shown in figure 1.

The time required to decompose most of the tartrate was similar when the cultures were grown in the tartrate broth at pH 4.5 and 5.0. Other test cultures also showed comparable rates of destruction. The very active group, under favorable conditions, can decompose all the tartrate (1 g per 100 ml) within 36 hours when ammonium *d*-tartrate is supplied, or 48 hours when calcium *d*-tartrate is used. The slow group needs from 5 to 14 days to decompose the tartrate supplied and may never cause complete destruction.

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[2] *Aerobacter aerogenes* has been found in larger numbers in commercial tartrate-bearing solutions with pH values of 3.5 to 3.6.

A characteristic which can be used for ready identification of the group of *Aerobacter aerogenes* which cause rapid decomposition of *d*-tartrate salts is their ability to produce copious amounts of gas in agar media containing these salts.

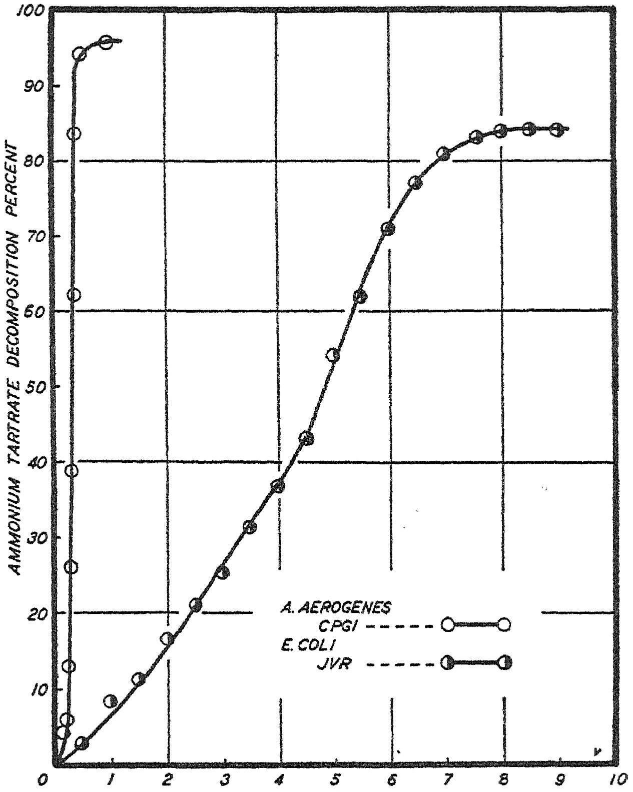

FIG. 1. THE DIFFERENCE IN RATE OF DESTRUCTION OF AMMONIUM TARTRATE BY A. AEROGENES (CPGI) AND E. COLI (JVR)

GROWTH OF COLIFORM BACTERIA IN DIFFERENTIAL TARTRATE MEDIA

Differentiation of species of *Salmonella* from other *Enterobacteriaceae* by the use of tartrate media has been suggested by numerous investigators including Altobelli (1914), Wagner (1920), Pesch (1921), Brown *et al.* (1924), and Jordan and Harmon (1928). Many strains representing different species of *Escherichia* and *Aerobacter* do not decompose tartrates. It has been claimed that tartrate media may be used for the differentiation of species of *Enterobacteriaceae* including

Escherichia coli. The medium of Jordan and Harmon has been used extensively with *Salmonella* and related bacteria. It was desirable to check the differential value of the two most promising media (Brown *et al.*, and Jordan and Harmon) when used with the cultures under investigation.

The media were prepared, inoculated, and incubated under conditions which duplicated as nearly as possible those prescribed by the original workers. The dehydrated Difco phenol red tartrate agar which duplicates the medium of Jordan and Harmon was also tested. Strains of the recognized species of *Escherichia* and *Aerobacter* which decomposed all or nearly all, about half, from one-fourth to one-tenth, and none of the tartrate, respectively, were tested. When the medium and technique of Brown *et al.* were employed, conflicting results were obtained. With test cultures which caused almost complete destruction of the sodium *d*-tartrate, positive qualitative tests for decomposition were obtained. With cultures which decomposed none or at most 15 to 25 per cent of the tartrate, negative results were obtained. When, however, cultures which had decomposed about half of the *d*-tartrate were used, obscure and inconclusive qualitative tests were observed. The results obtained with the Jordan and Harmon medium showed no significant difference in growth or reaction between strains of species of the two genera. The non-tartrate-decomposing strains could not be distinguished from tartrate-decomposing strains of *Escherichia* and *Aerobacter* when tested on Jordan and Harmon medium prepared from laboratory supplies or Difco dehydrated product.

It is certain that the proposed tartrate media cannot be used for differentiation of cultures of *Escherichia* and *Aerobacter*. The qualitative technique of Brown *et al.* is not delicate enough to detect small amounts of decomposition. The value of the Jordan and Harmon medium depends upon a change in reaction (pH). Tartrate decomposition by coliform bacteria is accompanied by an increase in pH to 8.0 or 8.5. Non-tartrate-decomposing strains liberate enough ammonia from the peptone to give corresponding increases in pH. It is obvious therefore that much of the data in the literature indicating tartrate decomposition by different bacteria is of questionable value.

THE TAXONOMIC STATUS OF BACILLUS TARTRICUS, AEROBACTER TARTARIVORUM, AND BACTERIUM SUCCINICUM

Three species of bacteria have been described whose validity is questionable in the light of our present knowledge of the coliform bacteria. These species are *Bacillus tartricus* (Grimbert and Ficquet, 1897), *Aerobacter tartarivorum* (Nijdam, 1907) and *Bacterium succinicum* (Sakaguchi and Tada, 1940). *Bacillus tartricus* and *Aerobacter tartarivorum* were given specific status primarily because of their ability to decompose *d*-tartrates. *Bacterium succinicum* was so named because it produced larger amounts of succinic acid from *d*-tartaric and other organic acids than did other coliform bacteria with which it was compared. These three species have characteristics which relate them to recognized species of coliform bacteria, as is seen in table 6. Furthermore, their characteristics have been investigated sufficiently to show their very close resemblance to the corresponding recognized species of the genera *Escherichia* and *Aerobacter*, as shown in the table.

It has been shown that the decomposition of *d*-tartrates is a characteristic common to some strains of all the accepted species of coliform bacteria belonging to the genera *Escherichia* and *Aerobacter*. It is not sufficient to claim specific status for any of the coliform bacteria because one strain produces more succinic acid from organic acids or glucose than other strains. It is well known that succinic acid is an end product arising from the decomposition of organic acids or glucose by members of the coliform bacteria.

TABLE 6

Probable synonymy of Bacillus tartricus, Aerobacter tartarivorum, and Bacterium succinicum

DESCRIBED SPECIES	BACILLUS* TARTRICUS	AEROBACTER† TARTARIVORUM	BACTERIUM‡ SUCCINICUM
Morphological characters			
Shape	short rods	short rods	short rods
Size (μ)	1–2 long	±1.2 x ±2.0	0.6–0.8 x 1.3–2.1
Flagella	+++	+++	Peritrichous
Spore formation	−(−)	−(−)	−(−)
Gram stain	−(−)	−(−)	−(−)
Biochemical characters, formation of			
Acetylmethyl carbinol (V.-P.) test)	+(+)	+(+)	−(−)
H_2S	0(−)	0(−)	+(+)
Indole	−(−)	+(+)	−(−)
Utilization of citric acid	0(+)	+(+)	+(+)
Methyl red test	0(+)	0(−)	+(+)
Gelatin liquefaction	+(+)	−(−)	−(−)
Acid and gas formation from			
Lactose	+(+)	+(+)	+(+)
Sucrose	+(+)	+(±)	+(+)
Starch	−(−)	+(+)	−(−)
Aesculin	0(+)	0(+)	0(−)
Salicin	0(+)	0(+)	−(−)
Glycerol	−(−)	+(+)	+(+)
Probable synonymy§	*Aerobacter cloacae* Bergey *et al.*	*Aerobacter aerogenes* (Kruze) Beijerinck	*Escherichia freundii* (Braak) Yale

0, not determined; −, negative reaction, + positive reaction, in original descriptions; presently accepted differential characteristics in ( ).

* Grimbert and Ficquet, 1897.

† Nijdam, 1907. +++ Type of flagellation not specified.

‡ Sakaguchi and Tada, 1940.

§ Consult table 1 and Bergey *et al.*, 1939; Levine, 1921; Levine, Epstein, and Vaughn, 1934; West, Gilliland, and Vaughn, 1941; Vaughn and Levine, 1942; and other literature for characteristics of these species.

Each group of bacteria having similar characteristics must bear only one name: the valid one based on prior use. It is suggested, therefore, that synonymy be created for *Bacillus tartricus*, *Aerobacter tartarivorum*, and *Bacterium succinicum* inasmuch as the possible synonymy of these organisms apparently has not received prior attention, except a suggestion by Barker (1936) on the possible synonymy of *Aerobacter tartarivorum* and *Aerobacter aerogenes*. On the basis of priority it is probable that *Bacillus tartricus* Grimbert and Ficquet is synonymous with *Aerobacter cloacae* (Jordan) Bergey *et al.*; *Aerobacter tartarivorum* Nijdam is synonymous with *Aerobacter aerogenes* (Kruze) Beijerinck; and *Bacterium succinicum* Sakaguchi and Tada is synonymous with *Escherichia freundii* (Braak) Yale in Bergey *et al.*

SUMMARY

The ability to decompose *d*-tartrate salts is possessed by some strains of all the recognized species of coliform bacteria.

The rates of destruction of *d*-tartrates by the coliform bacteria fell into two groups. One group represented by about half the cultures of *Aerobacter aerogenes* decomposed the *d*-tartrate salts rapidly and with vigorous evolution of gas. The rest of the cultures decomposed the *d*-tartrates slowly and with no visible evolution of gas.

Strains of *Aerobacter aerogenes* that cause very rapid destruction of *d*-tartrates bring about the most rapid and most complete destruction of *dl*- and *l*-ammonium tartrates. All species decomposed the *dl*- and *l*-ammonium tartrates less rapidly than the *d* isomer. The *l* form was attacked least rapidly of all.

The optimum temperature of incubation for maximum destruction of *d*-tartrates by the recognized species of coliform bacteria varied with the cultures investigated.

The initial pH of the medium has a marked effect on the ability of recognized species of coliform bacteria to grow and to decompose *d*-tartrate. Some of the species have definite optima ranging from pH 4.5 to 5.0; others have optima ranging between 5.0 and 7.3. Some strains of *Aerobacter aerogenes* decompose *d*-tartrates in media with initial pH values of 3.9.

Tartrate media used for the differentiation of *Salmonella* from other *Enterobacteriaceae* are of no value for distinguishing between cultures of *Escherichia* and *Aerobacter*.

The probable synonymy of *Bacillus tartricus*, *Aerobacter tartarivorum*, and *Bacterium succinicum* with *Aerobacter cloacae*, *Aerobacter aerogenes*, and *Escherichia freundii*, respectively, was discussed.

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METHODS OF STUDY OF ANTIPHAGE AGENTS PRODUCED BY MICROORGANISMS[1,2]

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Within recent years attempts have been made to determine whether microorganisms have the capacity of producing substances active against animal viruses in a manner comparable to the production of antibiotics or antibacterial agents (Jones *et al.*, 1945; Robinson, 1943). In this work the lack of techniques analogous to those commonly used for the study of antibiotics necessitated a more or less random choice of cultures. Needless to say, this indiscriminate testing would appear to be relatively inefficient as contrasted with the use of selective procedures such as those which have proved helpful in antibiotic work. In view of the increasing interest in the possibilities of an approach to the virus problem through studies of microbial antagonisms and the production of specific antiviral agents, attempts have been made to modify certain methods for investigating agents active against bacterial viruses or bacteriophages.

The practicability of utilizing bacteriophages as test agents in the search for chemotherapeutic substances active upon true viruses may be questionable in view of the marked differences between the bacteriophages and viruses, and even among members of each group with regard to their susceptibility to the direct action of chemical agents (Klein *et al.*, 1945). Nevertheless, any information concerning substances inhibitive to bacteriophages may contribute generally to knowledge of the intimate relationship between obligate intracellular parasites and their hosts. Moreover, experiments with bacterial viruses can be conducted with particular facility because of the ease and rapidity with which antiphage action can be detected and measured. In addition, the relative simplicity of the phage phenomenon, as compared with animal and plant virus systems, allows greater control of conditions.

The work reported here had as its objective the isolation of agents active against viruses, and is essentially quite distinct from studies on phage inhibition by bacterial extracts, which aimed at the elucidation of antigenic structure (Burnet, 1934) or the relationships and classification of bacteria (Levine and Frisch, 1933).

MATERIALS AND METHODS

Cultures of *Staphylococcus aureus* and *Escherichia coli* together with three bacteriophages employed in previous investigations (Jones, 1945) were used in

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[2] These investigations were supported by a grant from the Commonwealth Fund of New York.

these studies. Unless otherwise stated, 1.0 per cent nutrient agar was employed throughout. In the course of investigating various actinomycetes and fungi for antiphage action, an agar plate method for isolating microorganisms antagonistic to bacteriophages, agar streak and cup techniques to test such antagonists, and agar dilution and diffusion procedures for assaying cell-free preparations were developed. In view of the doubtful value of the soil enrichment procedure for the development of organisms antagonistic to bacteria (Waksman and Schatz, 1946), no studies were made concerning changes in the microbial population following the addition of bacteriophage to soil.

EXPERIMENTAL RESULTS

Several methods have been developed for the isolation of microorganisms antagonistic to bacteriophages and for the assay of antiphage activity. The first is similar to the bacterial agar method of Foster and Woodruff (1946) for the isolation of microorganisms antagonistic to bacteria. An agar medium seeded with the phage against which an antagonist is desired is employed for plating out a heterogeneous microbial flora, such as soil, compost, or manure, in sufficiently high dilutions to obtain well-isolated colonies. After a few days' incubation, the plates are coated with a thin layer of agar seeded with phage-susceptible bacteria, care being taken not to cause bacterial colonies to "run." For this purpose, an agar concentration of 0.5 per cent has been found satisfactory. If any of the organisms on the plates have produced diffusible antiphage agents, such colonies become surrounded by a zone of growth of the host cells; whereas at a distance from antagonistic organisms, as well as in the immediate vicinity of inactive colonies, the host cells will be lysed. Thus, with this method a zone of bacterial growth surrounding an antagonistic colony indicates antiphage activity, whereas in the corresponding technique involving bacteria-washed agar a clear zone of bacterial inhibition surrounding the antagonist is the criterion of antibacterial action (Waksman and Schatz, 1946).

The medium employed in the agar plate method affects greatly the practicability of the procedure. For example, nutrient agar, inoculated with suspensions of different soils and manures, allowed such rapid growth of spreading sporeformers that the host-cell-seeded agar, subsequently used to flood the plates, became contaminated. Acidified peptone glucose agar, which allows primarily the development of fungi, caused a rapid destruction of the phage. In general, neutral glucose asparagine 1 per cent agar has been found to be the most satisfactory medium so far tried.

In regard to the significance of phage antagonism, the fact must be appreciated that the elaboration of an antiphage agent is but one of the several possible mechanisms whereby the lytic action of phage can be inhibited. Consequently, the presence of a zone of host-cell growth is not absolute proof that the particular organism involved produces an antiphage substance. This has been shown to be the case with the corresponding plating procedure for isolating microorganisms antagonistic to bacteria. Actually, it has been found that many organisms, the original colonies of which showed good antiphage zones, produced no demonstrable antiphage action in the filtrates of different liquid media. It has also been observed that some organisms which were isolated from phage agar plates because of antibacterial zones surrounding the original colonies yielded broth filtrates active against phage but lacking demonstrable activity against the host cells (table 1). Some microorganisms isolated at random likewise produced liquid culture filtrates which inhibited bacteriophages but were without apparent effect upon host-cell growth. It would appear, therefore, that certain antibiotic agents in subinhibitive concentrations may prevent lysis by bacteriophages. This would indicate that the antiphage action of such substances was due to an effect on the host cells rather than directly upon the phages.

TABLE 1

Action of certain soil actinomycetes, isolated by the agar plate method, against Staphylococcus aureus phage K

CULTURE NO.	ORIGINAL COLONY ON GLUCOSE ASPARAGINE PHAGE AGAR	CULTURE MEDIUM*	AGAR TUBE TEST, FILTRATE DILUTION†		
			1:2	1:20	1:100
69	Antiphage	1	++++		
		2	++++		
		3	+++		
79	Antiphage	1	++++		
		2	+	++	
		3	+++		
87	Antiphage	1	+++		
		2	++	+++	++++
		3	+	++	++++
19	Antibacterial	1	+	++	
		2	0	+	+++
		3	0	+	++
56	Antibacterial	1	+	++	+++
		2	0	0	+
		3	0	0	+
74	Antibacterial	1	+++	++++	
		2	++	++++	
		3	++	++++	

* 1 = meat extract peptone; 2 = glucose tryptone; 3 = glycerol yeast extract. Each medium contained 0.2 per cent agar.

† Activity of filtrates of stationary cultures grown for 8 days at 28 C. ++++ = normal number of plaques; ++ = 10-fold reduction; + = at least a 100-fold reduction; 0 = no plaques.

Although it is possible to isolate microorganisms which produce antiphage filtrates in liquid cultures by the use of the phage-seeded agar medium, the limited data available (table 2) do not indicate any significant advantage for this procedure as contrasted with a random selection of organisms for testing. The relatively high percentage of active fungi, as compared with the actinomycetes (table 2), is very likely due to a pH effect in some cases, since the mold filtrates varied from pH 2.7 to 8.4. The tests with the two groups of organisms are not directly comparable for a number of other reasons, such as treatment of filtrates and phage exposure periods.

The agar streak method was employed for determining activity against different phages in a manner comparable to its use in the study of the antibacterial properties of microorganisms. For this purpose, the culture to be tested is streaked diametrically across a plate of agar seeded with phage. As in antibacterial studies, several different media are used. After appropriate incubation, cell-seeded agar is placed immediately adjacent to the growth of the antagonist, and the plate is then tipped so that the agar runs perpendicularly away to form a thin layer. After several hours' incubation, growth of the host cells occurs up to varying distances from the antagonist, depending on the degree to which the bacteriophage has been inhibited. Often, as shown in figure 1, there is an antibacterial zone within an antiphage zone. By the use of different phages, a spectrum of activity may be obtained. Some organisms are inactive by the streak test, although in the corresponding

TABLE 2

Antiphage activity of fungi and actinomycetes isolated at random and from glucose asparagine agar seeded with S. aureus phage K

SOIL SOURCE	ORIGINAL COLONIES	CULTURES TESTED	ACTIVE ORGANISMS, AGAR DILUTION TUBE TEST†
	Fungi*		
Enriched with fowl pox virus	Selected at random	8	7
Untreated	Selected at random	38	16
Untreated	Antiphage on phage agar	5	4
Untreated	Antibacterial on phage agar	9	7
	Actinomycetes‡		
Untreated	Selected at random	100	27
Untreated	Antiphage on phage agar	17	3
Untreated	Antibacterial on phage agar	10	2

* Grown in Czapek-Dox medium, glycerol nutrient broth, fungus broth, and corn steep liquor media for 10 days. Filtrates tested against a laboratory strain of *E. coli* phage and *S. aureus* phage K.

† Active organisms produced at least one filtrate active against one or more of the phages.

‡ Grown in media listed in footnote of table 1. Filtrates tested against laboratory strain of *E. coli* phage, *E. coli* PC phage, and *S. aureus* phage K.

agar-free medium they produce filtrates capable of inhibiting bacteriophages (table 3).

It is also possible to determine antiphage action by growing organisms in liquid medium in cylinders set on phage-seeded nutrient agar plates. After the desired period of incubation, the surface is carefully flooded with cell-seeded 0.5 per cent agar. If diffusible antiphage agents have been produced, the cup will be surrounded by a zone of host-cell growth. With this technique several organisms or different media can be tested on a single plate. Though the method works satisfactorily for actinomycetes, it frequently fails with certain bacteria which grow under the cup and spread out over the surface of the plate.

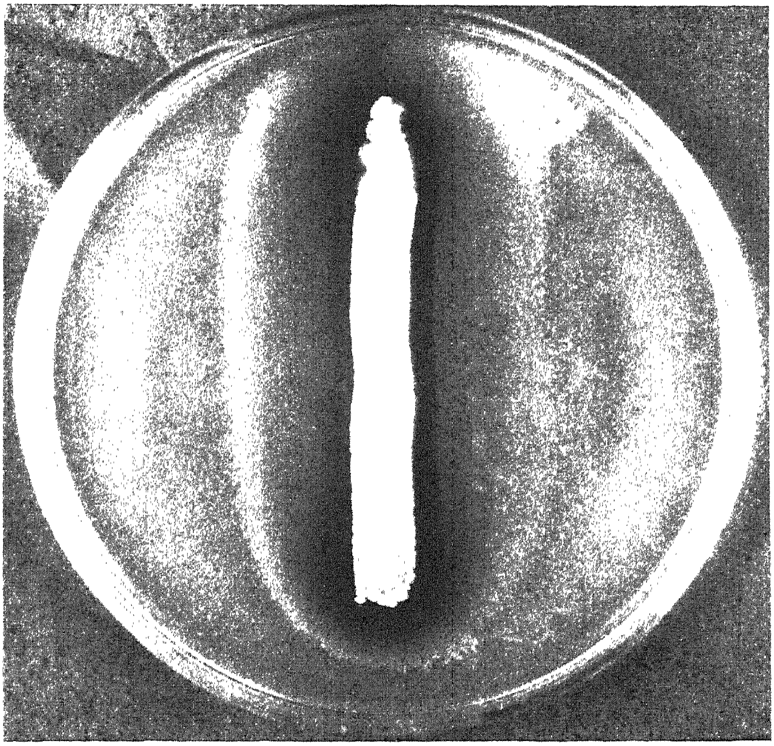

FIG. 1. STREAK TEST OF STREPTOMYCES NO. 193 AGAINST S. AUREUS PHAGE K ON TRYPTONE GLUCOSE AGAR

Zone of antiphage action surrounds inner zone of antibacterial action

In the agar dilution method, various dilutions of the preparations to be tested are placed in petri dishes and 10-ml portions of agar seeded with one or more phages are added. When culture fluids are tested, they may be sterilized by filtration or by heat treatment before use. After suitable incubation, individual areas of each plate are flooded with separate lots of agar, each seeded with a different bacterial host. The relative number of plaques is compared with the plaques on a control plate, and a spectrum of activity against different phages can thus be obtained.

A modification of this dilution procedure with only one phage can be conducted in test tubes. For this purpose, 1-ml portions of the phage suspensions and of the preparation being tested are mixed and incubated as desired. Subsequently, 3-ml portions of 1.7 per cent agar seeded with the bacterial host are added to each tube, which is then shaken and slanted; the higher agar concentration is used to compensate for the 2 ml of agar-free solution originally present. As in the foregoing method, the number of plaques per square centimeter is compared with the concentrations of plaques in the controls. In the present work, the potency of different filtrates was compared by taking as the end point that dilution which, when mixed with the phage overnight at 37 C, produced at least a 100-fold reduction in plaque titer.

TABLE 3

*Action of some soil actinomycetes against S. aureus phage K**

CULTURE NO.	MEDIUM	AGAR STREAK ZONE	AGAR TUBE TEST, CULTURE FILTRATE DILUTION		
			1:2	1:20	1:100
		mm			
56	1	31†	++++		
	2	16	+	++++	
	3	7	AB‡	++++	
79	1	0	0	0	++++
	2	2	0	0	++++
	3	3	++++		
87	1	0	++++		
	2	0	0	++++	
	3	2	0	++++	
97	1	0	+++		
	2	4	+	+	
	3	3	+	+	+
103B	1	0	+	++	
	2	16†	0	0	++
	3	15	0	+	++++

* See footnotes of table 1.
† Inner antibacterial zone present.
‡ AB = antibacterial action.

In the agar diffusion or cup test, the usual procedure is used for pouring plates of phage-seeded agar and for placing and filling the cylinders with the preparations to be tested. After incubation overnight, the plates are flooded with a thin layer of host-seeded agar. Where diffusible antiphage agents are present, the cups are surrounded with zones of bacterial growth, the host cells being lysed at a distance from the cups. With inactive preparations, complete lysis occurs right up to the cylinders. Figure 2 illustrates this technique. As has been found for the previous tests, increasing phage concentration yields progressively smaller zones of phage inhibition. The antibacterial zone in figure 2 indicates either that the antiphage agent is antibacterial in greater concentrations or that there is also present an antibiotic in addition to the antiphage agent.

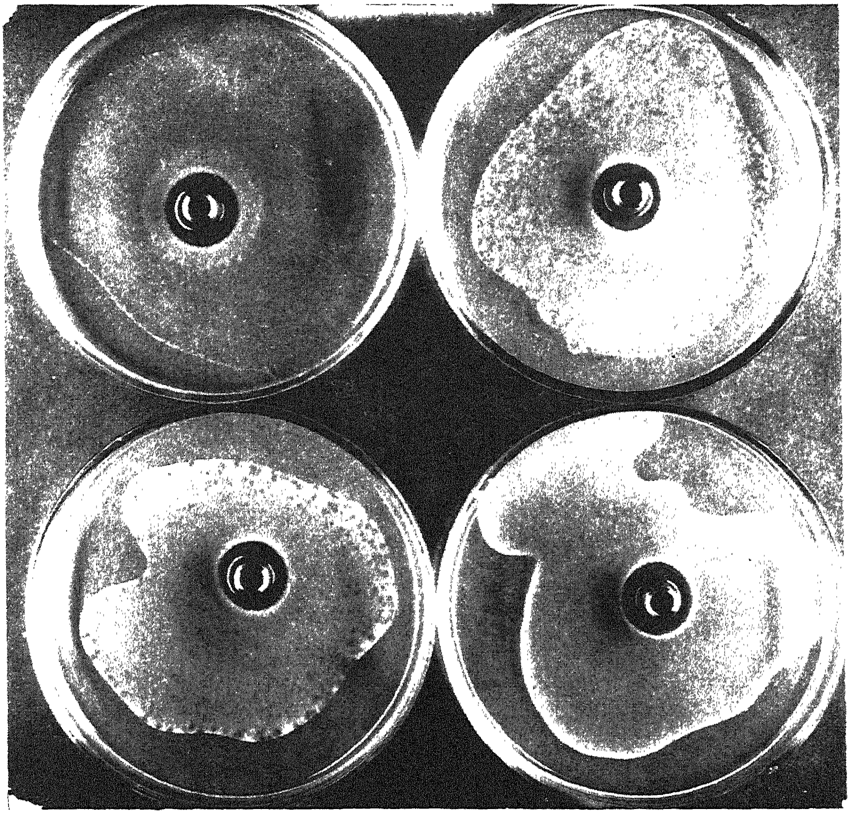

FIG. 2. AGAR DIFFUSION OR CUP TEST WITH THE ISOLATED AGENT OF STREPTOMYCES NO. 193 AGAINST THREE DIFFERENT CONCENTRATIONS OF S. AUREUS PHAGE K AND A PHAGE-FREE CONTROL

DISCUSSION

The various methods described have been developed for the study of agents active against bacteriophages. It is essential that the phages used be stable in the media over that period of time during which they are subjected to the metabolic products of the antagonists or to other preparations tested. In this respect, different phages, and possibly even different lots of the same phage, may vary considerably. It should be remembered that factors such as acid production may cause phage inactivation by the streak test, and that concentration of solvents, such as alcohol, must be considered in the agar cup tests.

In general, the techniques for the study of antiphage agents appear to possess inherent limitations which make their standardization more difficult than is the case with the analogous methods employed in antibiotic investigations. However, this may be expected in view of the greater complexity of the bacteriophage system. The antimicrobial tests involve (1) an antagonistic organism or an antibiotic substance, (2) a single medium, and (3) microorganisms whose growth is antagonized or inhibited. On the other hand, the system in which antiphage action is tested consists of (1) the antagonist or antiphage agent, (2) frequently two different media (one for plating and another for flooding the original agar layer with host cells), (3) a phage, and (4) the bacterial host of the phage. In addition, the time factor as well as other conditions of culture differ in the two systems. For example, the cup test for antiphage action requires a preincubation period to allow diffusion before the host cells are placed upon the surface of the phage-seeded agar. On the other hand, both cells and active agent are usually added simultaneously in the agar diffusion method for determining antibiotic action, the bacteria being present throughout the agar.

Any method for demonstrating and measuring the inhibition of phage action is considerably more complex in regard to the number and kinds of possible mechanisms which may be involved. Whereas an antibiotic must, of necessity, act directly upon the microbial cell, an antiphage agent may inhibit phage multiplication by acting directly upon the phage particle or by affecting in some manner the medium or host cell. With bacteriophage, the latter type of action may be due to the prevention of phage adsorption on, or entrance into, the cell; an alteration of host metabolism which renders impossible phage multiplication; or some other mechanism. It is, therefore, quite logical to anticipate difficulties in modifying for phage studies the procedures used for the isolation and study of antibiotic agents.

The limited data available indicate no particular advantage to the use of the agar plate method for isolating microorganisms antagonistic to phage. However, this very same comment has been made with respect to the bacteria-washed agar medium for the isolation of antibiotic-producing microorganisms, in which the criterion of activity has been the formation of lytic zones (Waksman and Schatz, 1946). Nevertheless, the selective streak and cup techniques for detecting and measuring antagonism and the agar dilution and diffusion method for the study of the agents themselves can be applied to phage investigations. Although some substances do not diffuse and as a result appear inactive by the cup procedure, such preparations can be satisfactorily handled by the agar dilution technique.

It is suggested that similar methods may possibly find application in the study of plant and animal viruses. It may be possible, for example, to test the infectivity of virus-seeded agar taken at various distances from a colony or a streaked organism, or from a cup containing a diffusible preparation.

SUMMARY

Methods useful in the screening of antagonistic organisms and studies of the antibiotic substances can also be applied to the isolation of microorganisms possessing antiphage properties and the study of the antiphage agents themselves. The resulting techniques, however, involve more variables than do the original and simpler procedures used in antibiotics.

The production of antiphage agents active against bacterial phages was investigated by the agar plate, agar streak, cup culture, agar dilution, and agar diffusion methods. The principle of all these methods is the ability of the virus host to grow on the medium in which virus action is inhibited. To what extent these methods or modifications of them can also be utilized in the study of substances active against plant and animal viruses remains to be determined.

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TEMPERATURE RELATIONS IN PHAGOCYTOSIS[1]

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The relation of fever and of subnormal temperatures to resistance and to well-being has been a matter of debate and varying practice for centuries. Since the dawn of history hot and cold topical applications and baths have had wide popularity, and antipyretic drugs have been used to a greater or less extent ever since their discovery.

In 1878 Pasteur, Joubert, and Chamberland noted that susceptibility to anthrax could be induced in fowl, animals normally resistant to this infection, simply by immersing them up to the thighs in a bath held at 25 C. The animals so treated suffered a reduction in temperature to 37 to 38 C and died of anthrax within 24 to 30 hours following inoculation with *Bacillus anthracis*, whereas the controls remained alive. Subsequently, Wagner (1890) confirmed their report, indicating further that "hypothermy diminishes the mobility and phagocytic functions of the leucocytes." When the animal's temperature was lowered, the tide of battle was turned against the leucocyte and the animal succumbed. Similar experiments with antipyretics were not so striking.

Modern interest in the therapeutic use of the febrile state was awakened by the report of Wagner von Jauregg (1918–19) that cases of tabes dorsalis and general paresis were improved following artificial infection with malaria. Although many of the physiological responses to fever have been clearly described, specific studies on the influence of fever on defense mechanisms have been few.

One of the most frequently cited reports is that of Rolly and Meltzer (1908), who studied in rabbits the effect of artificially increased temperature on resistance to infection, on phagocytosis, and on antibody production. Their figures, however, showed no greater differences than might be due to individual variation; in some of their experiments only two or three animals were used for a given procedure. Continuing the studies on phagocytosis, they reported that ingestion of several species of bacteria by human leucocytes *in vitro* was increased with temperature increases from 6 C to 39.5–40 C; beyond that point a decreasing activity occurred. The experiments with guinea pigs and guinea pig leucocytes gave irregular results.

Ellingson and Clark (1942), in a more complete study, disagreed with the findings of Rolly and Meltzer, especially in regard to the persistence and the production of antibodies. In rabbits already actively immunized against *Eberthella typhosa*, the induction of fever of 106 to 107 F (corresponding to about 41 C) was followed by rapid reduction of antibody titers as compared with controls with normal temperatures. In rabbits passively immunized with homologous antityphosus serum, fever of this degree accelerated the decline of antibody

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titers. Specific antibody responses were impaired when animals were subjected repeatedly to severe fever during the period of immunization. Their phagocytosis studies (*in vitro*) were few, but both with staphylococci and guinea pig leucocytes and with staphylococci and human leucocytes, an increase in the number of bacteria ingested was observed up to a temperature of 104 F or 40 C. Admittedly, this phase of their work was incomplete, and need for further study along this line was stressed by the authors.

Two other papers—one by Ledingham (1908), the other by Madsen and Wulff (1919)—are noteworthy in the field of phagocytosis-temperature relations. Ledingham observed enhanced phagocytosis with elevations in temperature from 18 to 42 C. He considered that this increased reaction was due to more adequate sensitization (i. e., sensitization in a much shorter time) at the higher temperature levels. Testing his hypothesis, he found that both opsonization and phagocytosis were favorably influenced, but opsonization to a more marked degree. He likewise noted that the rate of the reaction was involved rather than the end point.

Madsen and Wulff claimed that the optimum temperature for phagocytosis *in vitro* is that of the animal's body at the time the cells are withdrawn. If the temperature of the individual is normal, then maximum phagocytosis by that individual's cells should be at the normal body temperature for that particular animal species (around 37 C for humans, 39 C for guinea pigs and rabbits, and 41 C for fowls). Should the individual develop a febrile temperature, then optimum phagocytosis, according to these authors, would take place at that febrile temperature.

In attempting to analyze earlier phagocytic studies, it should be borne in mind that the lack of mechanical devices to ensure uniform mixing of phagocytic systems was characteristic of that period, so that chances for contact between the phagocytes and the bacteria were less and probably therefore did not so closely approximate those within the animal body.

A recent paper concerning phagocytosis-temperature relations is that of Cottingham and Mills (1943), who report experiments dealing with the effects upon phagocytic functions of polymorphonuclear leucocytes of vitamin-deficient white rats subjected to environmental temperatures of 68 F and 90–91 F. They concluded that phagocytic powers of deficient animals (a number of deficiencies were observed) were lessened regardless of environmental temperature. In their work, however, only 40 cells on each of two smears per animal per temperature were counted. In our experience, a count of 400 cells per animal per temperature is essential for a quantitative estimation of the extent of phagocytosis. These investigators went to the opposite extreme of that of the earlier workers and used extremely rapid shaking (560 reversals of direction per minute).

In view of the limitations of the earlier work and with the desire to pursue the observations of Ellingson and Clark, we undertook these studies on phagocytosis-temperature relations.

METHODS

The experiments reported here are divided into two parts: A, those concerned with phagocytosis over the range of 22 to 42 C, and B, those dealing with phagocytosis in the range of 37 to 45 C (with comparisons at 25 and 50 C).

A. Polymorphonuclear leucocytic exudation was induced in the peritoneal cavities of ten normal, mature guinea pigs, five normal, mature rabbits, and five normal, mature mice by the injection of starch aleuronat paste (3 per cent potato starch boiled in distilled water with 5 per cent aleuronat added) mixed with an equal volume of tryptose broth. The amounts injected were 10 to 20 ml in guinea pigs, 30 to 40 ml in rabbits, and 3 ml in mice.

After 18 to 24 hours the animals were exsanguinated by bleeding from the heart, and the serum so recovered was used in the phagocytic system. Various time intervals after injection of the irritant into mice were tested (4 to 24 hours) in an attempt to increase the yield of leucocytes, but consistent results were not obtained. Leucocytes were withdrawn from the peritoneal cavity after bleeding by washing out the area with a suitable amount of sterile physiological salt solution. The suspension was then centrifuged gently and standardized by means of the Neubauer counting chamber to 25,000 to 30,000 cells per mm^3. Leucocytes in all experiments were used within 4 hours after recovery from the test animal; the cells were retained at refrigerator temperature until ready for use, usually within an hour of their recovery.

The bacterial suspension employed was a laboratory strain of *Staphylococcus aureus* (hemolytic, mannitol-fermenting, and coagulase-positive) grown on tryptose agar for 18 to 24 hours, washed off with saline, shaken with sterile glass beads to break up the bacterial clumps, and standardized to McFarland $BaSO_4$ standard no. 1. In practice this suspension was diluted with an equal volume of saline just before its incorporation in the phagocytic system. The ratio of leucocyte to bacteria varied from 1:40 to 1:60. The standard bacterial suspension was discarded after a 6 weeks' period and a fresh emulsion prepared.

The leucocytes and serum were first pipetted into cleaned, sterile pyrex tubes (73 by 8 mm); the bacteria were added just before the mixture was incubated. One-tenth ml of each reagent was added; the tubes were sealed with paraffined corks and attached to the wheel of an electrically driven rotating device so constructed as to make 2.5 revolutions per minute about an axis 20 degrees from the long axis of the tube. This machine was also used by Ellingson and Clark (1942), but was modified so that the tubes containing the phagocytic system were rotated end over end. This modification was considered of greater value in the attainment of optimum contact between the leucocytes and the staphylococci. Five temperatures were included in each test: 22, 27, 32, 37, and 42 C. The temperatures of the water bath were carefully checked, and variations of more than 0.25 to 0.50 C were prevented.

After incubation for 10 minutes, the tubes were removed and 0.02 ml of the mixture placed on a slide and smeared over the surface with cigaret paper. After drying, the slides were stained in a dilute solution of Loeffler's methylene blue, the extent of dilution being determined separately for each experiment since the staining properties of leucocytes vary considerably from animal to animal. The use of this dilute stain permitted differentiation of the bacteria from the leucocytic nuclear material, which under these conditions stains less intensely.

On each of two smears for each temperature 200 leucocytes were examined for the presence of phagocytosis, making a total of 400 leucocytes observed at each temperature. The chief method of evaluation of the phagocytic reaction was that of Hamburger (1912), noting the percentage of active leucocytes; but a rough phagocytic index was also obtained according to the method of Leishman (1902), by recording the number of cells ingesting "few" (five or less) organisms and those phagocytizing "many" (more than five) bacteria. The labels of the slides were covered with tape and arbitrarily assigned a number by another person in order to rule out personal bias.

The body temperatures of the rabbits were recorded, and all showed normal temperatures. The temperatures of a representative sample of guinea pigs were taken and were found to be in the normal range. No attempt was made to obtain body temperatures of the mice (see figures 1, 2, and 3).

B. The procedure for the tests in the range of 37 to 45 C at 2-degree intervals (with experiments at 25 and 50 C for comparison) was the same as in the 22 to 42 C experiments with a few changes. The separate components of the phagocytic systems (serum, staphylococci, and leucocytes) were incubated for 1.5 minutes before being mixed at the temperatures at which they were to be rotated. Another variation was rotation of the tubes in the water bath for 5 minutes instead of 10. The staphylococci of this series only were maintained in a lyophilized state after preliminary tests had indicated little or no difference in the avidity with which these were ingested when compared with freshly prepared suspensions in systems containing the same leucocytes and serum. The body temperatures of the five guinea pigs from which cells and serum were obtained for this series varied from 39 to 40 C, a range considered normal (see figure 4).

PRESENTATION OF RESULTS

A. The results of representative phagocytic experiments in the guinea pig and rabbit groups are shown in figures 1 and 2. Actual counts are given: separate counts made by two individuals for the guinea pig group, those of a single observer for all other experiments. Variation in the evaluations by the investigators at a single temperature were 5 per cent or less except in a few instances. When great variation occurred, careful recounting was the practice and the discrepancies were ruled out mathematically.[2]

Figure 3 is a summary of the data from the guinea pig, rabbit, and mouse

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[2] Method for ruling out: adding together all the figures obtained at a given temperature except the one in question; averaging; finding the deviation of each figure from the average; averaging the deviations; multiplying the average deviation by the total number of determinations, including the questionable figure. If the figure obtained thus is less than the deviation of the doubtful figure from the average, then that determination may be discarded.

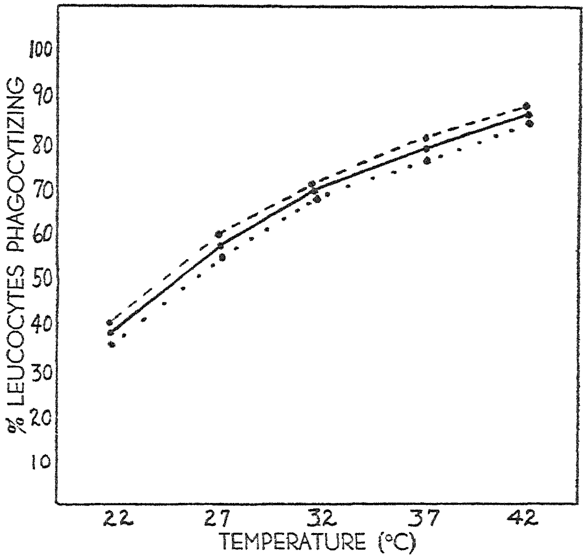

FIG. 1. REPRESENTATIVE GUINEA PIG EXPERIMENT
Top and bottom curves: results obtained by two individuals (D. H. and C. Z., respectively). Middle curve: average of the two counts

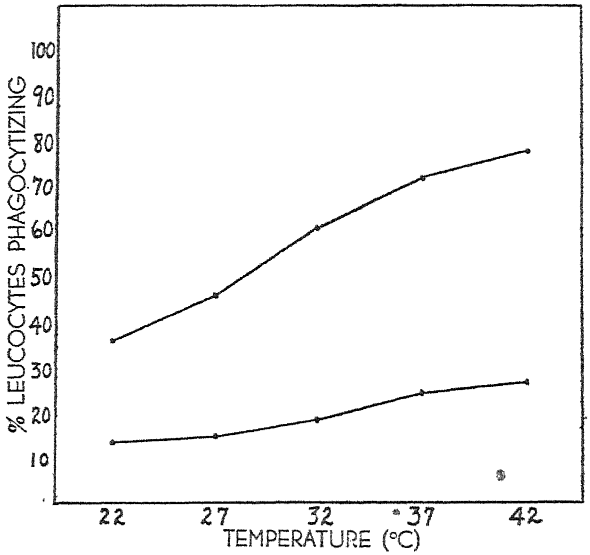

FIG. 2. REPRESENTATIVE RABBIT EXPERIMENT
Top curve: percentage of leucocytes phagocytizing. Lower curve: percentage of leucocytes ingesting "many" (i.e., more than five) bacteria

groups. The figures were obtained by designating the findings in each test at 22 C as unity (1.00) and comparing those at the four higher temperatures with this base. This is done for reasons of convenience and is intended merely to picture the average phagocytic ranges in each of the three groups.

Increases in phagocytic values with each rise in temperature (numerator = number of tests showing increase, denominator = number of tests in series) are shown below:

Guinea pig group:
- 22 to 27 C—9/10 (exactly the same value at the two temperatures was obtained in the other test)
- 27 to 32 C—9/10 (a significant decrease of 11 per cent occurred in the tenth test)
- 32 to 37 C—8/10 (insignificant decreases of 1 per cent and 3 per cent were observed in the other two tests)
- 37 to 42 C—8/10 (an insignificant decrease of 2 per cent appeared in one of the remaining two tests; there was a much greater decrease in the other, 12 per cent)

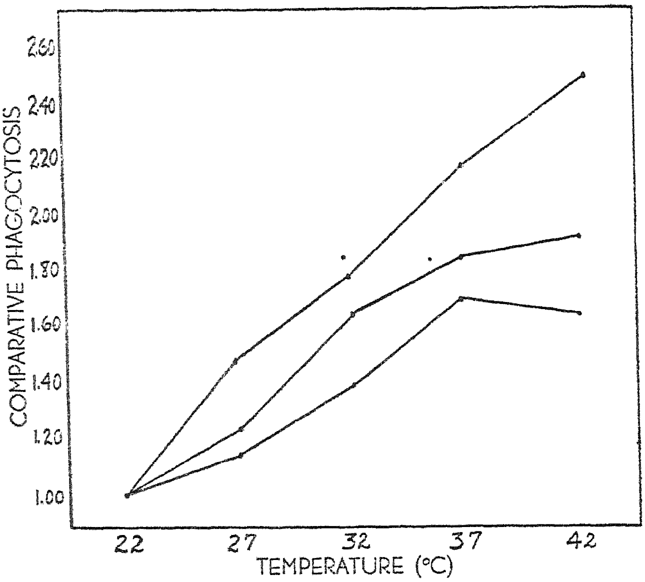

FIG. 3. PERCENTAGE OF LEUCOCYTES PHAGOCYTIZING IN EACH GROUP
Top curve: guinea pig group (average of ten). Middle curve: rabbit group (average of five). Bottom curve: mouse group (average of five)

Rabbit group:
- 22 to 27 C—5/5
- 27 to 32 C—5/5
- 32 to 37 C—4/5 (an insignificant decrease of 2 per cent occurred in the other)
- 37 to 42 C—4/5 (there was a significant decrease of 13 per cent in the fifth test)

Mouse group:
- 22 to 27 C—4/5 (a small decrease of 3 per cent was observed in the fifth test)
- 27 to 32 C—3/5 (the decreases in the remaining two tests were 1 per cent and 2 per cent, respectively)
- 32 to 37 C—5/5
- 37 to 42 C—2/5 (two tests gave small decreases of 3 per cent and 1 per cent, respectively, while in the other test the decrease was significant, 11 per cent)

B. The average comparative phagocytosis in the higher temperature range is shown in figure 4. Results were obtained by averaging the comparative phagocytosis values in the leucocyte-serum systems from the five guinea pigs at each temperature. Here, it will be noted, the comparative base (1.00) is placed at 25 C.

Increases in phagocytic values with each rise in temperature were as follows:

25 to 37 C—5/5

37 to 39 C—3/5 (of the remaining tests, one showed a decrease of 4 per cent at the higher temperature, but the other showed exactly the same value at both temperatures; two of the tests showing increases did so by only 1 per cent and are to be considered as the same as the lower temperature values)

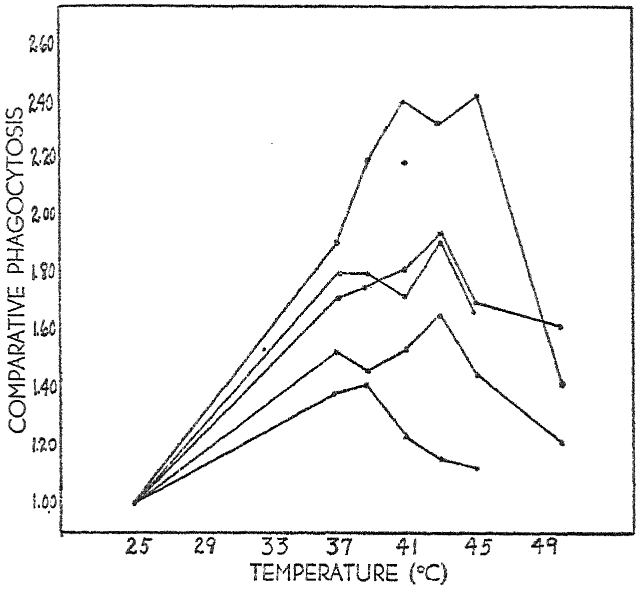

FIG. 4. PHAGOCYTOSIS AT 37 TO 45 C WITH COMPARISONS AT 25 AND 50 C
Each curve represents one complete experiment (guinea pig leucocytes and serum vs. staphylococci)

39 to 41 C—3/5 (one of the other tests gave a decrease of 3 per cent at 41 and the second, a decrease of 6 per cent; in one of the tests at which an increase occurred the amount of enhancement was only 1 per cent and is to be considered the same as at the lower temperature)

41 to 43 C—3/5 (decreases were of 3 per cent in each of the other two cases; increase in one experiment was an insignificant 2 per cent)

43 to 45 C—1/5 (decreases were all over 5 per cent except in one test where the drop was only 2 per cent; the one experiment giving an increase did so by 4 per cent)

45 to 50 C—0/5 (in two tests the leucocytes were too disintegrated to be examined. In two of the other 3 tests the drops were 28 per cent and 13 per cent, respectively; in the third the decrease was only 2 per cent)

DISCUSSION

It is readily observed that the over-all picture of phagocytosis *in vitro* by the exudative polymorphonuclear leucocyte in the guinea pig and rabbit groups is one of increase with each successive 5-degree rise in temperature over the range of

22 to 42 C. In the mouse group there was less phagocytosis at 42 as compared with 37 C, but this series of tests was unsatisfactory because of our inability to obtain a leucocytic exudation comparable both in quality and in quantity with those of the other two species. The irritant proved either too damaging, producing a sloughing of tissue cells, or too mild for a reaction of any kind. Four of the five experiments finally completed with any degree of technical parallelism with those of the other two animal groups displayed a range of phagocytosis well below that of the other species. The fifth mouse test, however, was technically comparable to the guinea pig and rabbit tests. Nevertheless, the results at each temperature in all five tests were averaged together to obtain the mouse group curve presented in figure 3. Because of the inadequacy of the mouse experiments our discussion is confined to the results obtained with guinea pigs and rabbits.

Although the lower temperatures are unphysiological for the animals employed in these tests, we considered it of importance to include them for the establishment of a curve. There is no doubt that phagocytosis increases, and at a consistent but somewhat decreasing rate, from 22 to 37 C. The next question obviously concerns the reaction between 37 and 42 C.

Two preliminary experiments were run with guinea pig phagocytic systems at the temperatures 37, 39, and 41 C. The first gave results varying only 1 per cent at the three temperatures; the second showed a decrease of 11 per cent and 10 per cent, respectively, at 39 and 41 compared with 37 C. For a temperature range containing 2-degree increments, an even more rigid technique than the one described must be employed. These increased precautions should include separate incubation of each component of the phagocytic system at the temperature at which the whole is to be tested, in order that incubation of the entire system may begin at the designated temperature instead of going through a brief period of adjustment. This condition was strictly adhered to in the five subsequent tests shown in figure 4. The arbitrary time of this preincubation was set at 1.5 minutes to avoid possible injury to the leucocytes. Here, it will be recalled, lyophilized organisms were used, and the period of rotation was shortened from 10 minutes to 5 minutes.

In figure 4 are the data, in comparative form, from the five experiments (guinea pig leucocytes and sera) within the range of 37 to 45 C with controls at 25 and 50 C. An increase appears in the percentage of actively phagocytic leucocytes beyond 37 C to about 43 C, followed by a decrease after this temperature. In only one test was there an enhancement at 45 C, and this rose insignificantly above the value obtained at 41 C. In this test (the topmost curve in figure 4), are observed a significant increase in phagocytosis from 37 to 41 C, a leveling off at 41, 43, and 45 C, and a decided drop at 50 C. In the other tests the fall occurred after 43 C, except in the experiment represented in the lowest curve in which 39 C was the high point. The decreases shown on the graph between 37 and 41 C, except possibly in the lowest curve, are considered of little, if any, significance (i.e., less than 5 per cent decreases in percentage of active leucocytes). The results at 50 C did not fall so low as those at 25 C in the three tests in which values could be obtained at that high temperature (in two experiments the white cells were too disintegrated to be counted). These results are interesting, for it appears that white cells can be subjected to temperatures higher than one observes even in patients with the severest febrile reactions and still give a good account of themselves—at least, *in vitro*. The white cells in the three experiments in which they could be counted at 50 C appeared to be morphologically as sound as those at the lower temperatures.

These results contradict the claims of Madsen and Wulff that optimum phagocytosis *in vitro* occurs at the temperature of the animal body at the time of withdrawal of the leucocytes, the only exception being shown in the lowest curve of figure 4 in which phagocytosis dropped sharply after 39 C, the approximate body temperature of the guinea pig contributing leucocytes and serum.

Fenn (1921–22) recalculated the results of Madsen and Watabiki (1919), who failed to determine a temperature coefficient in their studies. By calculation of the number of bacteria ingested per leucocyte per minute during the first half of the reaction, Fenn found that the rate of phagocytosis was almost a logarithmic function of temperature from 0 to 35 C and that Q_{10} was constant over that range and was equal to 2.0. His own experiments did not show comparable results, for he obtained an increase in temperature coefficient below 30 C and a lowered coefficient above 30 C. He considered that this increased coefficient at lower temperatures might be due to change of the leucocytic protoplasm from the *gel* to the *sol* state, a phenomenon easily effected by slight increases in temperatures in the lower range. The lower coefficient at higher temperatures, might, he thought, indicate that at higher temperatures, phagocytosis is dependent upon surface tension alterations.

By comparing the phagocytosis at each temperature with that at the next level, we observed a steady, progressive increase over the range of 22 to 42 C, the rate of this increase diminishing slightly at higher temperatures. Although our first tests (figure 3) did not establish a point of decline in the percentage of actively phagocytic cells, it seemed reasonable to assume it would not be far above 42 C, judging from the decrease in the rate of the reaction at the higher temperatures. This assumption was borne out in the tests pictured graphically in figure 4, in which the rate continued to decrease to the point of decline.

How the increase in the amount of phagocytosis can be correlated with the impairment of production and maintenance of antibodies at fever temperatures is not clear. It must be remembered, however, that the experiments of Ellingson and Clark on the effect of temperature on phagocytosis, as well as those presented here, were carried out *in vitro*, whereas their tests concerning antibodies were made *in vivo*. In the actual infectious process the leucocyte is subjected to elevated temperatures for longer periods than the arbitrary interval selected for these *in vitro* studies. It is entirely possible that the white cell becomes "weakened" in the febrile body before it comes in contact with the bacterial cell and so is less effective in combating invasion. Some information regarding this phase could be obtained by placing the polymorphonuclear cell at varying temperatures for different periods of time before running phagocytic tests *in vitro* with them. Repeated attempts on our part to develop a satisfactory technique *in vivo* for phagocytosis paralleling the tests *in vitro* were unfruitful because of difficulty in obtaining suitable leucocytic exudation to balance the bacterial suspension injected.

What, then, are the mechanisms involved in these phenomena of increase, on the one hand, and decline, on the other? What is the role of complement in these reactions? What are the physicochemical changes concerned? What part does mobility of the leucocytes play? What is the effect of the particular organism, *Staphylococcus aureus*, employed in these tests? Specifically, what is the influence of temperature on coagulase activator (Smith and Hale, 1944; Hale and Smith, 1945)? Are there changes in the metabolism of the leucocyte, and if so, are such changes a cause or an effect of increased ingestion? Is digestion of the organisms at higher temperatures likewise increased, or is it decreased, or does it remain fairly stable? It may be that the white cells are actually under adverse conditions at elevated temperatures and are artificially stimulated to an enhanced activity which lasts only during the short periods of these tests. During such stimulation the leucocyte may be unable to cope with the engulfed organisms, and when the initial stage has passed, the cells may begin to disintegrate, thus freeing organisms which have been damaged little or perhaps not at all. The accumulation of various metabolic products and the presence of leucocidin should likewise be kept in mind as possible participants in these involved processes. Obvious suggestions for future research, in addition to points already indicated, include investigation of different time limits, the effects of immune serum, the behavior of various types of organisms, and the activity of other types of phagocytes, particularly human cells. One must be aware also of possible physiological differences between blood leucocytes and exudative cells (Fleischmann, 1939). The crucial test regarding the practical value of increased temperature can be made only by use of *in vivo* procedures, and only then can parallels be accurately drawn. It is still unclear whether fever is advantageous or detrimental to the patient.

SUMMARY AND CONCLUSIONS

In a series of studies of phagocytosis *in vitro*, exudative guinea pig and rabbit polymorphonuclear leucocytes in a system with fresh homologous normal serum and *Staphylococcus aureus* showed enhanced phagocytic powers with 5 C increments within a temperature range of 22 to 42 C. These results are based on 10-minute incubation of the complete phagocytic system in a controlled water bath. Data here included are from the 15 tests (10 guinea pig and 5 rabbit) of which the techniques were the most satisfactorily standardized. In all, over 30 experiments were performed, and, regardless of variations in technique, the reaction showed the same trend.

Fifteen experiments with mice did not lead to clear-cut results; this was due to failure to secure an adequate leucocytic response. The results of the five tests in which there was a moderate amount of exudation are presented; they indicate an increased ingestion up to 37 C followed by a decline at 42 C.

In five tests with guinea pig leucocytes and fresh homologous normal serum (vs. *Staphylococcus aureus*) in which the temperatures ranged from 37 to 45 C with 2 C increments and with comparisons at 25 and 50 C, phagocytosis increased to a point approximating 43 C and declined rapidly beyond that point.

The increase in phagocytosis proceeded at a slightly decreased rate as elevated temperatures were reached.

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SALMONELLA SENEGAL, A NEW TYPE ISOLATED FROM A GREEN MAMBA SNAKE

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This new *Salmonella* type was isolated from the intestine of a female green mamba snake (*Dendraspis viridis*) which was submitted to the writers for autopsy by Mr. C. B. Perkins, Curator of Reptiles, San Diego Zoological Society. It was one of a pair purchased a few weeks previously for exhibition. The native habitat of the snake is western Africa from the Senegal to the Niger. Since the specimens had so recently been imported, the type name *senegal* was selected for this *Salmonella*. This snake died suddenly and had not been ill at any time following its arrival at the zoo. On autopsy it was noted to be poor in flesh, but not emaciated. The posterior intestine was studded with ulcers. Except for a slight congestion of the liver and mucosa of the stomach no other abnormalities were noted. The second snake is still alive and on exhibit.

Two strains each having biochemical and cultural characteristics of *Salmonella* were isolated from two different levels of the intestine. Other tissues examined (liver, heart, and lungs) were negative. The bacterium was a motile, gram-negative rod. It produced hydrogen sulfide but did not form indole, and it failed to liquefy gelatin in 60 days. Acid and gas were produced in 24 hours from arabinose, dulcitol, glucose, inositol, maltose, mannitol, rhamnose, sorbitol, trehalose, and xylose. Acid and gas were produced from cellobiose in 1 week by one strain, acid only by the other; and acid was produced from glycerol in 2 weeks. Lactose, raffinose, salicin, and sucrose were not fermented. All fermentation tests which were not positive after 72 hours were sealed and held at least 30 days at room temperature. The lactose tubes were held for 60 days and at no time showed any acidity (bromthymol blue). Sodium citrate and dextro-tartrate were utilized. Litmus milk became very slightly acid in 24 hours and neutral in 72 hours, and after 1 week was alkaline.

Antigenic analysis by the method of Edwards and Bruner (1942) showed that its somatic antigen was identical to that of *Salmonella rubislaw* (XI). Alcoholized antigens were agglutinated to titer with *S. rubislaw* antiserum. Absorption of this serum with *S. senegal* removed all the somatic agglutinins for the homologous strain. Antiserum prepared by injection of a rabbit with boiled antigen of one strain of *S. senegal* reacted to titer (2,560) with an alcoholized *S. rubislaw* antigen. Likewise, absorption of the *S. senegal* antiserum with *S. rubislaw* removed all the "O" agglutinins for both types. Therefore the somatic antigen for *S. senegal* is XI.

Preliminary examination of the flagellar antigens showed that the culture was diphasic. Serums were prepared for both phases after they were separated by the method of Edwards and Bruner (1942). Phase 1 was agglutinated to the titer of, and removed all H agglutinins from, serum prepared from phase 1 of *S. rubislaw* (r). Likewise, phase 1 of *S. rubislaw* was agglutinated to the titer of, and removed all agglutinins from, serum derived from phase 1 of *S. senegal*. Phase 1 of *S. senegal* is r.

Phase 2 of *S. senegal* was agglutinated by serums for all the nonspecific phases of the genus. When tested with single factor serums, it was agglutinated only by serum for factor 5. Serum prepared for phase 2 of *S. senegal* was exhausted of H agglutinins by absorption with phase 2 of *Salmonella manhattan* (1,5 . . .). Phase 2 of *S. senegal* removed all agglutinins from serum derived from phase 2 of *Salmonella cholerae-suis* (1,5 . . .). Phase 2 of the new type is 1,5 . . . .

The diagnostic formula of *S. senegal* is XI:r, 1,5 . . . .

ACKNOWLEDGMENTS

We wish to acknowledge with appreciation the co-operation of Mr. C. B. Perkins, Curator of Reptiles, San Diego Zoological Society, for furnishing the snake and historical data; Mr. T. J. Taylor, University of California, for technical assistance; and Dr. P. R. Edwards, National Salmonella Typing Center, Lexington, Kentucky, for his aid in verifying our results.

SUMMARY

A new *Salmonella* type isolated from a green mamba snake (*Dendraspis viridis*) is described. It has the antigenic formula XI:r,1,5 . . . ., and has been designated *S. senegal*.

REFERENCE

Edwards, P. R., and Bruner, D. W. 1942 Serological identification of *Salmonella* cultures. Kentucky Agr. Expt. Sta., Circ. 54.

THE EFFECT OF AERATION ON AMYLASE PRODUCTION BY BACILLUS MACERANS

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During the course of an investigation on the cultural conditions affecting production of amylase by *Bacillus macerans*, it was observed that aeration resulted in an increase in amylase yields and in an appreciable decrease in the time of incubation necessary for its production.

A 5 per cent rolled oats medium buffered with 2 per cent calcium carbonate, recommended as most suitable for amylase production by Tilden and Hudson (1942), was employed. The sterile medium was inoculated with a heavy saline suspension of a 24-hour culture of *B. macerans* (Sch. no. 4) grown on nutrient agar containing 1 per cent glucose. Incubation was at 37 C. The time of incubation varied with the conditions of the experiment. Aeration was accomplished by passing sterile air, saturated with water vapor, through aloxite aerator stones. Control cultures prepared and inoculated under the same conditions were incubated without aeration for the same period of time. Representative samples of the cultures were removed aseptically for analysis after various periods of incubation. The samples were filtered through infusorial earth pads on a Buchner funnel to remove slime and solid materials. The clear filtrate was analyzed for amylase content by use of the microscopic iodine test described by Tilden and Hudson. The enzyme unit employed was that arbitrarily defined by the authors as that amount of enzyme present in 1 ml which will convert 30 mg of starch to the "brown violet stage" in 30 minutes at 40 C. In preparations in which the enzyme content was very low, a 0.5 per cent starch and a 1/60 N iodine solution were used. Fermentations were conducted in quadruplicate. The results recorded in the accompanying tables are average values.

TABLE 1

Effect of aeration on production of amylase by Bacillus macerans (Sch. no. 4)

TIME OF INCUBATION	AMYLASE CONTENT (UNITS PER ML)	
	Aerated	Unaerated
days		
4	0.2	0.2
7	3.8	1.2
14	4.5	1.9
21	5.0	2.2

Table 1 shows that the amylase content of aerated samples after 1 week of incubation exceeded that of unaerated cultures after incubation for 3 weeks.

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In confirmation of these findings, similar experiments with six other strains of *B. macerans* (table 2) showed that all strains tested produced more enzyme in a shorter period of time when the cultures were aerated.

TABLE 2

Production of amylase by various strains of Bacillus macerans after 1 and 3 weeks' incubation

STRAIN NO.	AMYLASE CONTENT (UNITS PER ML)		
	Aerated 1 week	Unaerated	
		1 week	3 weeks
E.V.I.-231	3.5	0.2	2.2
277-S	4.5	1.0	1.9
277-T	2.7	0.2	2.1
888	5.3	2.4	2.3
588	2.7	0.4	1.7
583	5.5	0.9	2.4

SUMMARY

By the use of aeration it was found possible to increase the yields of *Bacillus macerans* amylase and to decrease appreciably the incubation period required for its production.

REFERENCE

TILDEN, E. B., AND HUDSON, C. S. 1942 Preparation and properties of the amylases produced by *Bacillus macerans* and *Bacillus polymyxa*. J. Bact., **43**, 527–544.

ACETIC ACID INHIBITION OF GRAM-NEGATIVE BACILLI IN CULTURE MEDIA

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Many substances have been added to culture media in attempts to inhibit the growth of gram-negative bacilli, especially those of the coli-proteus-pyocyaneus group, thereby facilitating the isolation of gram-positive organisms (Floyd and Dack, 1939; Snyder and Lichstein, 1940; Lichstein and Snyder, 1941). However, with effective concentrations of these substances, many of the gram-positive bacteria are also inhibited, their colonial characteristics are altered, or typical hemolysis is suppressed or disguised by changes in the medium. Further, in liquid media, the gram-negative bacilli soon outnumber the gram-positive organisms and, when the culture is plated, the appearance of isolated gram-positive colonies is more a matter of luck than of good management. Sporulating forms may be isolated by the use of heat, but the cocci are often exceedingly difficult to obtain in pure culture.

For some years it has been the practice of clinicians to irrigate with dilute acetic acid such wounds as are contaminated or infected with *Pseudomonas aeruginosa* or related species in attempts to decrease the activity of these organisms. Levine and Fellers (1939, 1940), studying the effect of acetic acid on microorganisms involved in the spoilage of food, demonstrated that the toxic effect of this substance is due in part at least, to the undissociated molecule and not entirely to the changes in the hydrogen-ion concentration.

This laboratory has been engaged in the study of the bacterial flora of traumatic and surgical wounds and has found the isolation of gram-positive organisms from gram-negative overgrowths a frequent and discouraging problem. Therefore, an assay of the efficacy of acetic acid as an inhibitory agent for gram-negative organisms was made.

Technique. Meat tubes, consisting, on an average, of about 1 inch of ground beef heart in 15 ml of beef heart infusion broth, are boiled in a water bath and cooled rapidly in running water. Glacial acetic acid is diluted to 10 per cent and 1 per cent in sterile distilled water and added to the meat tubes as follows:

Tube 1—0.1 ml of 1 per cent
Tube 2—0.5 ml of 1 per cent
Tube 3—0.1 ml of 10 per cent
Tube 4—0.5 ml of 10 per cent
Tube 5—1.0 ml of 10 per cent

The tubes are shaken slightly to distribute the acid, which forms a heavy, white precipitate in the broth. Each tube is then inoculated with 0.1 ml of an 18- to

24-hour meat tube culture of the specimen under study and incubated at 37 C, aerobically or anaerobically according to the preference of the organisms sought. The cultures are examined microscopically at 24-hour intervals, and, when good growth of the gram-positive or moderate to poor growth of the gram-negative organisms is observed, the material in that tube is streaked on horse blood agar plates (plus 5 to 6 per cent of 95 per cent ethyl alcohol if the flora includes *Proteus*). After overnight incubation aerobically and anaerobically at 37 C, the plates are examined for colonies of the desired types.

Results. Table 1 gives the typical pH determinations in the series of meat tubes after the addition of acetic acid. The pH was determined by the use of BDH universal indicator.

The results obtained by the use of this method are typified by the following summarized protocols:

Case 1, chronic leg ulcer: Staphylococci and streptococci were seen microscopically in meat tube cultures but were overgrown by *P. aeruginosa* when the tubes were plated out. Beta hemolytic streptococci, group C Lancefield, and coagulase-positive hemolytic *Staphylococcus aureus* were isolated from acetic acid tubes 3 and 4, thus accounting for all morphologic types seen with the microscope.

TABLE 1

Changes in the pH of meat tubes upon addition of acetic acid

TUBE NO.	pH BEFORE ADDITION OF ACID	AMOUNT OF ACID ADDED	pH IMMEDIATE	pH AFTER 18-HOUR INCUBATION UNINOCULATED
		ml		
1	7.5	0.1 of 1 per cent	6.5	6.5–7.0
2	7.5	0.5 of 1 per cent	6.0	6.5
3	7.5	0.1 of 10 per cent	5.0–5.5	6.0
4	7.5	0.5 of 10 per cent	4.5	5.0–5.5
5	7.5	1.0 of 10 per cent	4.0	4.0–5.5

Case 2, chronic draining sinus from perisplenic abscess: Staphylococci and streptococci were seen microscopically but were overgrown by *P. aeruginosa* and *Escherichia coli* on plates. Coagulase-positive *S. aureus*, coagulase-negative *Staphylococcus albus*, aerobic and anaerobic nonhemolytic streptococci, and *Fusobacterium* sp. were isolated from tubes 3, 4, and 5.

Case 3, chronic leg ulcer: Streptococci, seen in smears from meat tubes, were overgrown by *Proteus*, even on 5 per cent alcohol plates. Alpha hemolytic streptococci were isolated on alcohol blood agar plates streaked from tube 5.

Case 4, perineal abscess: The very heterogeneous flora was overgrown by the abundant *E. coli* present in the culture. Alpha hemolytic and nonhemolytic streptococci, *Clostridium welchii*, and *Clostridium bifermentans* were isolated from tubes 3, 4, and 5, the clostridia without the necessity of resorting to the application of heat. These organisms accounted for all the morphologic types seen in microscopic preparations of the culture.

Case 5, cystitis in a debilitated patient: Streptococci and spore-bearing bacilli, seen microscopically, were overgrown by *E. coli* and *P. aeruginosa* in the cultures. Nonhemolytic streptococci, *Fusobacterium* sp., and *C. welchii* were isolated from tubes 1, 2, and 3, again without the necessity of heating to obtain the clostridium.

Case 6, persistent sinus from osteomyelitis of ribs: Streptococci and staphylococci were overgrown by *P. aeruginosa.* Coagulase-positive hemolytic *S. aureus* and beta hemolytic streptococci, group A Lancefield, were isolated from tube 3.

Case 7, abscess of the upper arm: Staphylococci and streptococci were overgrown by *Proteus* even on 5 per cent alcohol blood agar plates. Anaerobic nonhemolytic streptococci, coagulase-positive hemolytic *S. aureus*, and coagulase-negative *S. albus* were isolated when material from tube 3 was streaked on alcohol plates.

As can be seen from the foregoing cases, a number of strains, interesting from a clinical point of view, can be recovered with the aid of this medium. Various species of clostridia have been isolated from anaerobic blood agar plates streaked directly from acetic acid medium. Some species of clostridia also sporulate well in this medium and can be recovered with greater ease by heating these cultures to 80 C for 20 minutes than by similarly heating meat tube cultures to which acid had not been added.

In approximately one-third of the specimens inoculated into this medium, staphylococci, seen microscopically in the original tubes, were inhibited at the concentrations necessary to suppress the gram-negative bacilli and could not be recovered by this method. Other staphylococci and the great majority of the streptococci encountered were easily isolated.

Several specimens were inoculated directly from the lesions into acetic acid meat tubes to ascertain whether primary culture in the acid-containing medium would further facilitate the isolation of the gram-positive organisms. No growth occurred in any of the tubes containing the acid, however, and direct inoculation was abandoned in favor of 18-hour incubation in routine media before transfer to the acetic acid tubes.

In order to be certain that growth in the acetic acid medium combination did not change any characteristic for which the organism might be tested in this laboratory, strains isolated without resort to this technique were grown in the medium, recovered by plating, and tested against the original strains as controls. In no case was any change observed in (1) color, hemolysis, or coagulating power of the staphylococci, (2) type of hemolysis of the streptococci, or (3) Lancefield grouping or *in vitro* virulence test (Ward and Lyons, 1935) of beta hemolytic streptococci.

It was found that tubes 1 and 2 (0.1 and 0.5 ml of 1 per cent acetic acid) contained concentrations usually too low to inhibit the gram-negative bacilli, and that tubes 4 and 5 (0.5 and 1.0 ml of 10 per cent) had concentrations usually too high to allow any bacterial growth. These tubes, therefore, are omitted unless demanded by the reactions of a particular culture.

The technique (using 0.1 and 0.2 ml of 10 per cent acetic acid) is now routinely employed in this laboratory whenever this type of mixed culture is found and has proved to be a simple, inexpensive, and very valuable aid, especially in the isolation of the gram-positive cocci.

A few experiments were done using HCl and H_2SO_4 in parallel with acetic acid, but these were entirely unsuccessful.

SUMMARY AND CONCLUSIONS

The addition of acetic acid to meat tubes has been found to be a valuable aid in the isolation of gram-positive bacteria from cultures overgrown by gram-negative bacilli of the coli-proteus-pyocyaneous group. The method, which is practical for routine laboratories, is described, and a number of typical cases are cited.

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STUDIES ON THE PRODUCTION OF ANTIBIOTICS BY ACTINOMYCETES AND MOLDS

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Many important diseases have been brought under control in recent years through rapid advances in the field of chemotherapy. A large number of agents antagonistic to pathogenic bacteria have been obtained from microorganisms. Some of these, penicillin for example, have been found to possess remarkable therapeutic properties. Others, such as streptomycin and streptothricin, (Waksman *et al.*, 1944; Jones *et al.*, 1944) appear promising as agents for combating disease organisms. But despite the fact that progress has been made in the treatment of certain diseases, there are some caused by bacteria, molds, and other agents which have not responded to the chemotherapeutic substances now in use. For this reason a continued search for antibiotic agents of therapeutic value seems justified.

Waksman and coworkers (1941) demonstrated that antagonistic actinomycetes are prevalent and widely distributed in nature; 106 of 244 actinomycetes isolated at random were found to be antagonistic to *Bacillus subtilis*. Seventy-seven of 164 actinomycetes were found to be active against *Staphylococcus aureus*.

Alexopoulos (1941) performed experiments to determine how widespread are *Actinomyces* substances which are toxic to fungi. A total of 80 *Actinomyces* strains were studied; 45 of these inhibited the growth of the test fungus, *Colletotrichum gloeosporoides*, but 35 had no effect. A series of studies on antibiotic production by fungi has been reported by Wilkins and Harris (1942, 1943*a*, 1943*b*, 1944). Of the first 100 fungal species studied, 40 per cent of the *Aspergillus* species and 25 per cent of the *Penicillium* species gave positive results against one of the test organisms, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas pyocyanea* (*P. aeruginosa*). In an examination of another 100 fungal cultures these authors found that penicillia and aspergilli were the most promising. Of 33 penicillia tested, 12 were active against *E. coli*, 31 against *S. aureus*, and 7 against *P. pyocyanea*. Of all the cultures examined by these authors, they found 50 per cent of the aspergilli and 50 per cent of the penicillia to be active; whereas 30 percent of the basidiomycetes and no phycomycetes or ascomycetes showed activity. Surveys of antibiotic production by molds were reported by Atkinson (1943*a*, 1943*b*, 1943*c*) and Atkinson *et al.* (1944). Of one group of 68 penicillia tested, 18 showed some activity against bacteria. In another survey 50 penicillia and 2 aspergilli were studied; 15 of the penicillia and aspergilli were found to inhibit bacterial growth.

In 1944 studies were begun in this laboratory on the isolation and testing of actinomycetes and molds for antibiotic production. The studies reported in this paper deal with screening tests conducted on approximately 1,000 molds and actinomycetes that were isolated from soils collected at many points throughout this country.

METHODS

The methods employed in this survey were adapted from those of other investigators. For a description of methods used in searching for antibiotic substances, see the recent book by Waksman (1945).

Isolation of cultures. Cultures of molds and actinomycetes were isolated from the soil and obtained in pure culture before tests were made for antibiotic activity. Soil samples were collected in Michigan, Texas, Florida, Oklahoma, California, Arizona, Louisiana, North Carolina, Mississippi, Georgia, Tennessee, New Mexico, and South Carolina. A total of 239 soil samples were studied; 1,007 actinomycetes and 221 molds were isolated. Members of the *Mucorales* and rapidly growing species of the Fungi Imperfecti were ignored. Isolations of molds were confined to aspergilli, penicillia, and a miscellaneous group of Fungi Imperfecti. In selecting colonies of actinomycetes for isolation an effort was made to isolate from each plate only those colonies differing in appearance.

The agar media used for the isolations were as follows: (1) peptone yeast extract glucose agar at pH 7.0, (2) Czapek-Dox agar at pH 6.5 to 7.0, and (3) sodium caseinate glucose salts agar at pH 7.0. Media (1) and (2) were used for molds and (3) was used for actinomycetes.

Screening actinomycetes and molds for antibiotic activity. In screening cultures for antibiotic activity it is desirable to have methods that are rapid and sensitive. After investigations of some of the methods used for screening organisms for antibiotic production, it was decided to use both a primary and a secondary screening. The first screening was used (1) to determine what cultures were active, (2) to get information concerning the extent of the activity, and (3) to get information concerning the groups of organisms against which the cultures were active. The secondary screening was used mainly to select cultures for further studies, and, since various media were used, information was obtained concerning media requirements for antibiotic production.

Primary screening. The streak plate method was used in the primary screening. Single, large colonies of the actinomycetes and molds were grown on agar plates that contained 20 ml of agar. The media employed were as follows:

1.	Difco beef extract	4.0	g
	Difco peptone	4.0	g
	NaCl	2.5	g
	Glucose	10.0	g
	Solubilized liver	1.0	g
	Difco yeast extract	1.0	g
	Agar	20.0	g
	Dist. H_2O	1,000.0	g
2.	Dextrin	10.0	g
	Difco tryptone	5.0	g
	K_2HPO_4	2.0	g
	NaCl	2.0	g
	$FeSO_4 \cdot 7H_2O$	0.01	g
	Agar	20.0	g
	Dist. H_2O	1,000.0	ml
3.	Brown sugar	10.0	g
	$NaNO_3$	3.0	g
	KH_2PO_4	1.0	g
	$MgSO_4 \cdot 7H_2O$	0.5	g
	$FeSO_3 \cdot 7H_2O$	0.01	g
	KCl	0.5	g
	Difco yeast extract	2.0	g
	Agar	20.0	g
	Dist. H_2O	1,000.0	g

4.	Corn steep	10.0 g
	Lactose	20.0 g
	$NaNO_3$	3.0 g
	KH_2PO_4	0.50 g
	$MgSO_4 \cdot 7H_2O$	0.25 g
	$ZNSO_4 \cdot 7H_2O$	0.02 g
	Agar	20.0 g
	Dist. H_2O	1,000.0 ml

5.	Difco peptone	1.0 g
	Difco yeast extract	1.0 g
	Glucose	10.0 g
	Agar	20.0 g
	Dist. H_2O	1,000.0 ml

All media were adjusted to pH 6.5 to 7.0 before autoclaving. Media 1 and 2 were used for actinomycetes. Media 3 and 4 were used for molds when screening them against bacteria, but media 4 and 5 were used when screening them against pathogenic fungi.

In conducting the screening tests, colonies 20 to 25 mm in diameter were prepared by growing the cultures on these media for 4 to 7 days at 23 C; however, with some molds it was necessary to make tests earlier on account of spreading growth. After sufficient growth of the molds and actinomycetes had occurred, test organisms were streaked from the edge of the test colony to the edge of the agar plate. The plates were incubated and observations made for inhibition of growth. In the case of bacteria, five organisms, representative of various morphological and physiological types, were streaked on each plate from broth cultures. The following bacteria were used: *Bacillus subtilis* (laboratory strain), *Staphylococcus aureus* FDA 209, *Escherichia coli* ATCC 26, *Proteus vulgaris* ATCC 8427, and *Pseudomonas aeruginosa* ATCC 9027. The plates were incubated at 37 C for 24 hours.

The following pathogenic fungi were selected for these studies: *Trichophyton gypseum* ATCC 9533 (*T. mentagrophytes*), *Candida albicans* ATCC 2091, *Sporotrichum schenkii* ATCC 7968, *Blastomyces dermatitidis* ATCC 7967, *Endodermophyton tropicale* ATCC 4568 (*T. concentricum*), and *Cryptococcus hominis* ATCC 1655 (*C. neoformans*). These fungi were selected for study because they represent different morphological types, and they represent groups causing a wide variety of infections, both systemic and cutaneous. Both yeasts and Fungi Imperfecti are included in this group. In making streak tests against these organisms, spore suspensions were prepared from agar slants and streaks were made on the test plates, which were incubated at 30 C for 40 hours, then read to determine whether inhibition of growth had occurred.

Secondary screening. Of the promising cultures from the primary screening 38 molds and 107 actinomycetes were tested against bacteria, whereas 35 molds and 58 actinomycetes were tested against a fungus.

Cultures were grown at 23 C in duplicate 500-ml Erlenmeyer shaker flasks containing 75 ml of medium, and the resulting fluids were tested for antibiotic activity by tube dilution methods. Samples were taken from the culture flasks with sterile 6-mm glass tubes fitted with cotton filters to remove mycelium. In the case of actinomycetes tests were made after 3, 5, and 7 days of growth, and the molds were tested after 4 and 6 days of growth. A variety of culture media were used in order to increase the possibilities of finding promising antibiotic producers and as an aid in determining whether an antibiotic was a new one. Molds were grown on the following media:

1.	Corn steep	30.0 g
	Lactose	20.0 g
	Tap water	1,000.0 ml
	pH—4.2 to 4.5	
2.	Corn steep	30.0 g
	Glucose	20.0 g
	Tap water	1,000.0 ml
	pH—4.2 to 4.5	
3.	$NaNO_3$	3.0 g
	K_2HPO_4	1.0 g
	$MgSO_4 \cdot 7H_2O$	0.5 g
	$FeSO_4 \cdot 7H_2O$	0.01 g
	Brown sugar	20.0 g
	Tap water	1,000.0 ml
	pH 6.2 to 6.6	
4.	Difco peptone	10.0 g
	Difco yeast extract	1.0 g
	Glucose	10.0 g
	NaCl	2.0 g
	Tap water	1,000.0 ml
	pH 6.5 to 7.0	

The following media, all of which were adjusted to pH 6.9 to 7.1, were used for actinomycetes:

1.	Difco tryptone	5.0 g
	K_2HPO_4	2.0 g
	NaCl	2.0 g
	$FeSO_4 \cdot 7H_2O$	0.01 g
	Glucose	10.0 g
	Dist. H_2O	1,000.0 ml
2.	Difco peptone	4.0 g
	Difco beef extract	4.0 g
	Wilson liver fraction L	1.0 g
	Difco yeast extract	1.0 g
	Glucose	10.0 g
	Dist. H_2O	1,000.0 ml

3. Same as medium 2 except starch replaced glucose.

4. Same as medium 2 except lactose replaced glucose.

5.	Corn steep	20.0 g
	Glucose	10.0 g
	Dist. H_2O	1,000.0 ml
6.	Smaco casein hydrolyzate	3.0 g
	Glucose	10.0 g
	Dist. H_2O	1,000.0 ml

The culture fluids were tested for antibiotic activity against the bacteria, *Escherichia coli* and *Staphylococcus albus*, and against the fungus, *Cryptococcus hominis*. In conducting the tests against the bacteria, dilutions of the culture fluids were made in the following medium: peptone (7.5 g), yeast extract (2.5 g.), distilled water (1,000.0 ml), pH 7.25. On the first day of testing the following dilutions of the culture fluids were made: 1:10, 1:30, 1:100, 1:300, and 1:1,000. Tubes, containing 10 ml of medium each, were inoculated with 0.1 ml of a 24-hour broth culture of the test organism; they were incubated for 16 to 18 hours at 37 C and read to determine the highest dilution of the culture fluids that prevented growth. If the 1:1,000 dilution of a culture fluid completely inhibited growth of a test organism on the first day of assay, higher dilutions were run on the next day; these dilutions were generally as follows: 1:300, 1:1,000, 1:3,000, 1:10,000, and 1:30,000.

Tests against *C. hominis* were made in a medium of the following composition: $(NH_4)_2SO_4$ (1.0 g), glucose (20.0 g), yeast extract (5.0 g), and distilled water

(1,000.0 ml). The pH was adjusted to 7.5. The same system of dilutions was used for these tests as for those against the bacteria. The inoculum was prepared in flasks shaken for 24 hours at 23 C. Each tube containing 10 ml of medium was inoculated with 0.1 ml of culture. Tubes were incubated for 24 hours at 30 C.

RESULTS AND DISCUSSION

Primary screening. A total of 734 actinomycetes and 210 molds were tested against the five bacteria in the primary screening; 382 actinomycetes and 117 molds showed some inhibitory activity against at least one of the test organisms. A total of 764 actinomycetes and 315 molds were tested against the six pathogenic fungi; 350 actinomycetes and 178 molds inhibited at least one test organism. From these figures it is seen that 53 per cent of the cultures were active against at least one test bacterium, and 49 per cent were active against at least one test fungus.

TABLE 1

Scores made by actinomycetes and fungi in primary screening

BACTERIA AS TEST ORGANISMS			FUNGAL PATHOGENS AS TEST ORGANISMS		
Score	Per cent actinomycetes	Per cent fungi	Score	Per cent actinomycetes	Per cent fungi
0	47.5	46.0	0	53.7	42.5
1 to 3	27.7	15.7	1 to 3	16.1	20.7
4 to 6	13.3	28.6	4 to 6	11.9	13.0
7 to 9	4.9	3.8	7 to 9	10.5	13.3
10 to 12	4.0	5.7	10 to 12	5.0	7.0
13 to 15	2.6	0.0	13 to 15	1.6	2.9
			16 to 18	0.7	0.6

The results of the primary screening are presented in an abbreviated form in table 1. A scoring system was used to compile the data shown in this table. Each mold or actinomycete was scored on the basis of the medium on which it performed best. A culture was given 3 points for each test organism that it completely inhibited, 2 points for strong inhibition, and 1 for slight inhibition. Since there were 5 test bacteria, a mold or actinomycete would make a score of 15 if it completely inhibited the growth of all 5; but a score of 18 was possible against the fungi, as 6 of these were used. In the table are found the percentages of molds and actinomycetes that made particular scores against the test organisms. This table serves to illustrate the order of activity against the two groups of organisms, bacteria and pathogenic fungi.

Figures 1 and 2 show the relative sensitivities of the test organisms to the molds and actinomycetes, and they also give the orders of activity exhibited by the molds and actinomycetes against the bacteria and pathogenic fungi. It is noted that *B. subtilis* was the most sensitive bacterium to the action of both actinomycetes and molds; it was inhibited by more than 50 per cent of the cul-

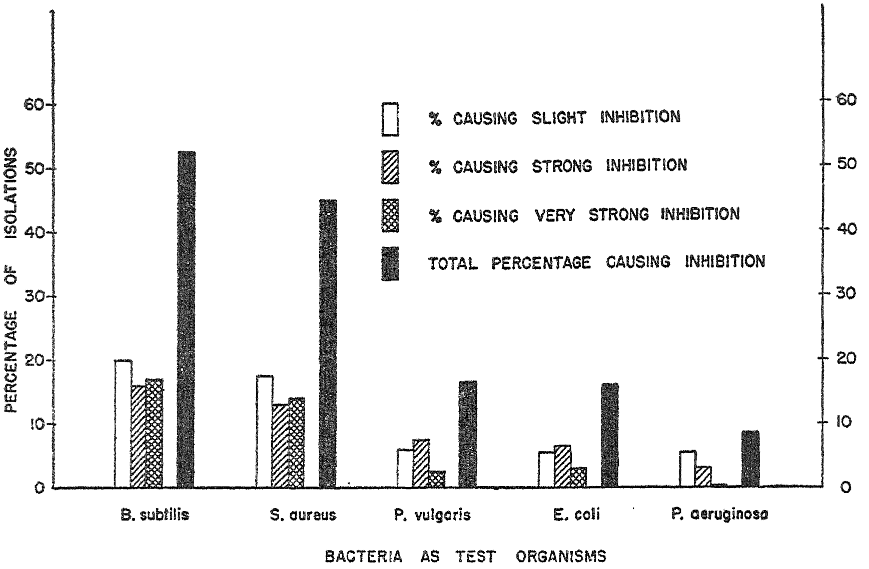

FIG. 1. THE DEGREE OF ANTIBIOTIC ACTIVITY OF ALL FUNGAL AND ACTINOMYCETE ISOLATIONS AGAINST EACH OF FIVE TEST BACTERIA

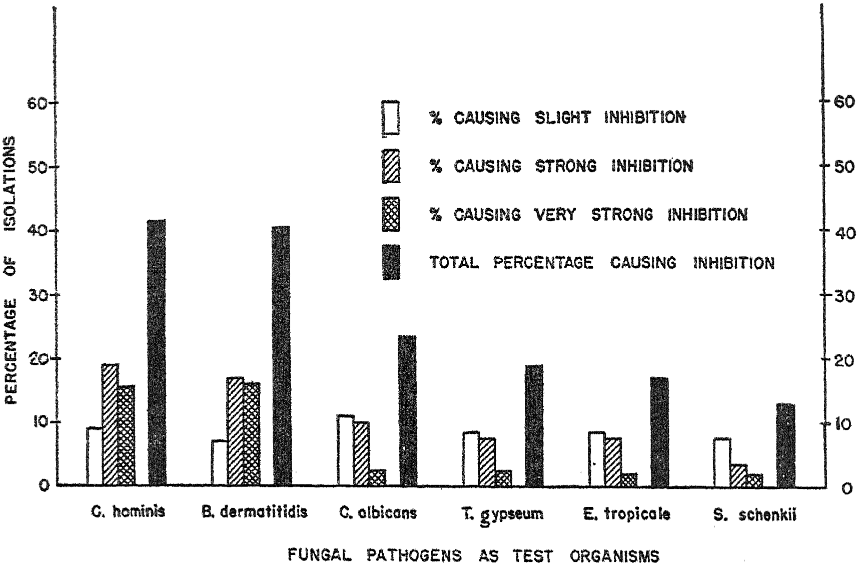

FIG. 2. THE DEGREE OF ANTIBIOTIC ACTIVITY OF ALL FUNGAL AND ACTINOMYCETE ISOLATIONS AGAINST EACH OF SIX TEST FUNGI

tures. *S. aureus* was only slightly less sensitive than *B. subtilis*. *P. aeruginosa* was the most resistant of the test bacteria, being inhibited by 10.9 per cent of the actinomycetes and 5.3 per cent of the molds. Approximately 14 per cent of the actinomycetes inhibited the growth of *P. vulgaris* and *E. coli*, whereas 18 per cent of the molds inhibited these cultures.

C. hominis, inhibited by 41.0 per cent of the cultures, was the most sensitive organism among the pathogenic fungi; it was more sensitive to actinomycetes than to molds. *B. dermatitidis* was the most sensitive of the pathogenic fungi to molds, but it was more resistant to actinomycetes than was *C. hominis*. *T. gypseum* was the most resistant fungus to the action of the actinomycetes; it was inhibited by 11 per cent of these cultures. *S. schenkii*, inhibited by 10 per cent of the molds, was the most resistant fungus to this group of organisms.

TABLE 2

Distribution of antibiotic activity of actinomycetes and molds according to genus

GENUS	TESTED AGAINST BACTERIA			TESTED AGAINST FUNGAL PATHOGENS			TESTED AGAINST BOTH BACTERIA AND FUNGI: NUMBER ACTIVE AGAINST				
	Number active	Number inactive	Number tested	Number active	Number inactive	Number tested	Bacteria only	Molds only	Both	Neither	Number tested
Streptomyces	399	370	769	353	399	752	149	109	233	237	728
Micromonospora	1	2	3	0	6	6					
Nocardia					8	8					
Total for actinomycetes	400	372	772	353	413	766	149	109	233	237	728
Aspergillus	28	24	52	43	23	66	6	11	22	13	52
Penicillium	89	51	140	124	49	173	21	34	67	15	137
Other genera of hyphomycetes	11	47	58	13	68	81	6	5	3	43	57
Total for fungi	128	122	250	180	140	320	33	50	92	71	246
Total for actinomycetes and fungi	528	494	1,022	533	553	1,086	182	159	325	308	974

Table 2 gives the distribution of antibiotic activity of the actinomycetes and fungi according to genus. Most of the actinomycetes belong to the genus *Streptomyces*. It was found that 52 per cent of the *Streptomyces* strains were active against at least one test bacterium. Three *Micromonospora* strains were tested; one of these showed activity. Forty-seven per cent of the *Streptomyces* strains were active against the fungi. Six *Micromonospora* and 8 *Nocardia* strains were tested against the fungi, but none inhibited their growth. One may also observe in table 2 that 32 per cent of the *Streptomyces* strains that were tested against both bacteria and fungi were active against both groups, but 33 per cent were not active against either group.

Slightly over 50 per cent of the aspergilli inhibited growth of the test bacteria, and 65 per cent were active against the fungi. Sixty-four per cent of the penicillia were active against the bacteria, and 70 per cent were active against the fungi. Forty-two per cent of the aspergilli that were tested against both bacteria and fungi inhibited the growth of both groups, but 25 per cent were not active against either group. Sixty-seven per cent of the penicillia were active against both bacteria and fungi, but 11 per cent were not active against either group. This activity against both bacteria and fungi may be attributed to the production of a single antibiotic, active against both bacteria and fungi; or it may be due to the production of more than one antibiotic. Also, when evaluating the significance of the percentage of active cultures, it must be borne in mind that one active species may have been isolated many times.

Secondary screening. Since many antibiotic-producing cultures were found by the primary screening, some of the promising cultures from the primary screening were studied by the secondary screening method. A total of 107 actinomycetes and 37 molds were tested against bacteria by the secondary screening method. Fifty-eight actinomycetes and 35 molds were tested against the fungus, *C. hominis*. The results of these studies are presented in table 3; the numbers of cultures that inhibited the test organisms at particular dilution ranges are given. These dilutions represent the highest ones at which complete inhibition of growth occurred on any of the days the culture fluids were tested. Since too much space would have been required to present these data in detail, there is no indication of the relative merits of the different culture media. Suffice it to say that no medium proved generally superior to the others for antibiotic production. However, for any particular actinomycete or mold there were usually great differences in the antibiotic potencies produced in the different culture media.

TABLE 3

Potencies of culture fluids produced by molds and actinomycetes*

CULTURES TESTED	TEST ORGANISM	DILUTIONS				
		No inhibition	1:10 to 1:100	1:100 to 1:1,000	1:1,000 to 1:10,000	>10,000
107 Actinomycetes	*E. coli*	27	20	24	29	7
	S. albus	6	18	24	43	16
37 Molds	*E. coli*	27	10	0	0	0
	S. albus	24	12	1	0	0
58 Actinomycetes	*C. hominis*	1	9	14	24	10
35 Molds	*C. hominis*	2	11	9	8	5

* The figures represent the numbers of cultures that produced culture fluids inhibiting the test organism in the particular dilution ranges shown. These figures represent the maximum potencies of the cultures.

It is observed that 80 of the 107 actinomycetes produced culture fluids that inhibited the growth of *E. coli*, and 101 produced fluids that inhibited *S. albus*. It is worthy of note that 29 actinomycetes produced culture fluids that prevented the growth of *E. coli* in dilutions of 1:1,000 to 1:10,000, whereas the maximum dilution for complete suppression of growth for culture fluids of 7 actinomycetes was greater than 1:10,000. The results against *S. albus* are even more striking, for 43 of the 107 actinomycetes yielded culture fluids that completely inhibited growth of this organism in dilutions of 1:1,000 to 1:10,000, and 16 cultures produced fluids that prevented growth in dilutions above 1:10,000.

The results obtained with molds were not so encouraging as those obtained with actinomycetes. Only 10 of 37 molds produced culture fluids that inhibited the growth of *E. coli*, whereas 13 produced fluids that inhibited *S. albus*. No mold culture fluid inhibited *E. coli* in a dilution greater than 1:100 and *S. albus* in a dilution greater than 1:1,000. It is entirely possible, however, that further work on the molds will lead to the development of conditions more suitable for antibiotic production.

All except 1 of 58 actinomycetes produced culture fluids that prevented growth of the fungus, *C. hominis*, and all but 2 of 35 molds produced culture filtrates that suppressed growth of this organism. It is noted that culture fluids from 24 of 58 actinomycetes prevented growth in dilutions of 1:1,000 to 1:10,000, and 10 cultures yielded fluids that prevented growth in dilutions above 1:10,000. Eight of 35 molds gave culture fluids that prevented growth in dilutions of 1:1,000 to 1:10,000, and 5 gave fluids that prevented growth in dilutions greater than 1:10,000.

Since many molds and actinomycetes were found to produce antibiotic substances, there are a number of cultures available for further studies. The more promising cultures are being investigated to determine whether the antibiotic substances produced by them are of any therapeutic value. Studies are being conducted on the microbiology, chemistry, and pharmacology of the antibiotic substances.

SUMMARY

Molds and actinomycetes were isolated from the soil and studied for antibiotic activity. Approximately 1,000 cultures were tested.

The cultures were tested against a group of bacteria and a group of pathogenic fungi by the streak plate method. Approximately 50 per cent of the cultures were found to produce inhibitory substances.

A number of cultures that performed well in the primary screening (agar streak) were studied further in shaker flasks. Thirty-seven molds and 107 actinomycetes were grown in this way, and their resulting culture fluids were tested against *Escherichia coli* and *Staphylococcus albus*; 58 actinomycetes and 35 molds were tested against *Cryptococcus hominis*. Ten molds and 80 actinomycetes produced culture fluids that inhibited the growth of *E. coli*, whereas 101 actinomycetes and 13 molds produced culture fluids that inhibited the growth of *S. albus*. All but 1 of 58 actinomycetes produced culture liquors that prevented the growth of *C. hominis*, and all but 2 of 35 molds yielded fluids that prevented growth of this organism.

ACKNOWLEDGMENT

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THE USE OF SOLUBLE STARCH MEDIUM IN THE PREPARATION OF SMOOTH "O" SALMONELLA ANTIGENS

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One of the chief difficulties that we have encountered in working with the *Salmonella* group of organisms has been in obtaining smooth "O" antigens from stock strains for the preparation of antisera, and also from cultures which have been submitted for identification. These were sufficiently numerous to warrant an investigation into the problem.

It was noted by Arkwright (1921) that bacterial cultures, particularly members of the enteric group, were subject to changes in colony morphology; that the variants of this group were characterized by the appearance of irregular rough colonies on solid media; and that, when grown in broth, the growth was granular and had a tendency to settle to the bottom of the tube. In many cases spontaneous agglutination occurred in the presence of 0.85 per cent sodium chloride. This S to R variation takes place frequently under the ordinary laboratory methods of cultivation, either by successive transfers in liquid media or after repeated subculture on solid media over a period of years. Serial transfer of rough cultures through a motility tube does not noticeably reduce the roughness of the culture.

The appearance of colonies on solid medium, however, is not always a reliable indication of antigenic composition, as was pointed out by Edwards and Bruner (1942), since "O" antigens of cultures that give a granular growth in broth are sometimes still recognizable, whereas cultures that produce colonies on solid media that are smooth in appearance may yet elaborate antigens which are so changed that they cannot be identified.

It seemed desirable to study first the effect on the "O" antigen of growing the culture on media of varying hydrogen-ion concentrations. To this end separate amounts of veal agar were prepared, and the pH of each was adjusted to form a series from 7.0 to 9.0. After sterilization the pH readings were 6.5 to 8.5.

Three *Salmonella* cultures were selected—a rough strain, a smooth strain, and one which was partially rough. These were each planted on a series of veal agar slants. After 18 hours' incubation at 37 C, individual "O" antigens were prepared from each of the cultures, using the method recommended by White (1927) for the preparation of "O" antigens from cultures that are slightly rough. In this method the growth from one agar slant is suspended in 1.0 ml of absolute alcohol and heated at 60 C for 1 hour. The organisms are then sedimented by centrifugation, the alcohol decanted, and the bacilli resuspended in 0.5 ml of normal saline. These antigens were tested for specific smooth "O" agglutination by the spot-agglutination technique recommended by Edwards and Bruner (1942).

It was found that the pH of the medium only affected slightly the roughness of the antigens, but that when rough cultures were grown on a medium with a final pH of 7.5, while the antigen still showed nonspecific granulation, they agglutinated slightly in the smooth homologous antiserum. It was also noted that the smooth "O" antigen agglutinated more satisfactorily in its homologous antiserum when the cultures were grown on veal agar slants with a final pH of 7.5.

From the detailed studies of White (1929) it appears that there is good reason for believing that the surface of the bacterial cell contains a polysaccharide component which is shared by certain types. In the rough variant this normal smooth polysaccharide antigen is lost, and other nonspecific antigenic components are exposed. These also include a polysaccharide, which differs from the one which characterizes the normal smooth antigen, and, in addition, two other components that are apparently protein in nature. By further variation this second polysaccharide antigen also may be lost, and the antigen be dominated by the two protein antigens, which White has named ρ_1 and ρ_2.

It was suggested by one of us (L. B.) that the addition of starch to the culture medium might have the effect of reversing the S to R variation, and also of preserving cultures in the smooth state.

Three *Salmonella* cultures (*S. london*, *S. illinois*, and *S. paratyphi* A) which showed varying degrees of roughness were each grown on veal agar to which had been added 1, 2, or 5 per cent soluble starch. "O" antigens were prepared by the method of White (1927), and tests were made by the spot-agglutination technique against antisera for the smooth "O" antigens of heterologous and homologous strains. The results of these tests are shown in table 1.

Almost all the cultures showed some improvement after one subculture on 1 per cent or 2 per cent starch. If the antigen from the first subculture still showed nonspecific granulation, or failed to agglutinate in antiserum for the smooth "O" antigen of the homologous strain, a further subculture on starch was made. On repeated subculture, 2 per cent starch gave better results than did 1 per cent starch. When 5 per cent starch was used, the growth on the slant was considerably reduced in amount and was mucoid in appearance.

The media used in all susequent tests was veal agar with 2 per cent soluble starch. The method of preparation is as follows:

Veal infusion agar

Veal infusion broth	1,000 ml
Neopeptone (Difco)	10 g
Sodium chloride	5 g
Agar (Difco)	22.5 g

Boil 30 minutes in a double boiler, stirring frequently. Adjust the pH to 8.2. Filter through cotton and cheesecloth. Sterilize in the autoclave for 20 minutes at 15 pounds of pressure. Blow a bulb on a 6-inch tube inside a 300-ml flask. Place 100 ml of distilled water in the flask, and 20 grams of soluble starch (Lintner) in the bulb. Sterilize in the autoclave for 20 minutes at 15 pounds of pressure. Cool veal infusion agar and starch to 40 C. Break the bulb and disperse the starch in the distilled water. Add the starch solution to the veal infusion agar. Mix thoroughly. Dispense in 5-inch and 7-inch tubes, and slope.

Stock cultures which had become rough were given serial subculture on this medium. "O" antigens were prepared after each transfer and tested against antisera specific for smooth "O" antigens, including antiserum for the homologous smooth strain. The results of these tests are shown in table 2.

TABLE 1

The effect of varying amounts of starch in veal agar in the preparation of somatic antigens

	FIRST SUBCULTURE		FURTHER SUBCULTURE	
	Smooth "O" antisera			
S. minnesota	Heterologous antisera	XXI, XXVI	Heterologous antisera	XXI, XXVI
Veal infusion agar	g	+		
Veal infusion agar 1% starch	–	++++		
Veal infusion agar 2% starch	–	++++		
Veal infusion agar 5% starch	growth unsatisfactory			
S. illinois	Heterologous antisera	XXXIV	Heterologous antisera	XXXIV
Veal infusion agar	g	g++	g	g+++^{3}
Veal infusion agar 1% starch	g	g++	sg	sg+++^{3}
Veal infusion agar 2% starch	sg	sg++++	–	++++^{3}
Veal infusion agar 5% starch	growth unsatisfactory			
S. newington	Heterologous antisera	III, XV	Heterologous antisera	III, XV
Veal infusion agar	G	G	G	G^{4}
Veal infusion agar 1% starch	G	G	g+	g+++^{4}
Veal infusion agar 2% starch	G	G	–	++++^{4}
Veal infusion agar 5% starch	growth unsatisfactory			

G = heavy nonspecific granulation; g = slight nonspecific granulation; sg = very slight nonspecific granulation; – = smooth, no granulation, no agglutination; +, ++, +++, ++++ = amount of specific agglutination; numerals indicate the number of further subcultures.

It will be noted that some cultures responded more readily than others, but in almost every case the nonspecific granulation was reduced, and some agglutination was visible with antiserum for the smooth homologous strain after one subculture on starch medium. All cultures produced smooth "O" antigen after serial transfer on this medium. The number of subcultures required varied with the degree of roughness in the original culture. Starch medium was also used for cultures received for identification that were showing S to R variation. With two exceptions these responded much more readily than had the stock strains. The majority produced a smooth "O" antigen after one subculture. Five cultures required further transfer, but all produced smooth "O" antigen after serial subculture.

In some cases, particularly in that of cultures requiring a number of subcultures before they produced a completely smooth "O" antigen, there was a tendency for the cultures to revert to the rough form when stored in the cold room. This was especially noticeable in a strain of *S. paratyphi* A. When first taken from stock, it was rough, showing very irregular colonies on solid medium and a granular growth in broth. It showed no sign of specific agglutination in antiserum for the smooth "O" antigen of *S. paratyphi* A. It was subcultured three times on veal agar with a pH of 7.6. The culture showed slight improvement, and agglutination of its somatic antigen was just visible in smooth "O" antiserum for *S. paratyphi* A. It was then subcultured serially on starch agar. The cultures became smooth, showed no nonspecific granulation, and agglutinated well in "O" antiserum for the smooth strain. This culture, now smooth, was sealed and stored in the cold room. It was tested at 4-week intervals. At the end of 8 weeks it was again rough.

TABLE 2

Effect of 2 per cent starch in veal agar in the preparation of smooth "O" antigens from stock cultures which had become rough

	"O" ANTIGEN FROM VEAL AGAR		"O" ANTIGEN FROM 2% STARCH 1ST SUBCULTURE		"O" ANTIGEN FROM 2% STARCH 2ND SUBCULTURE		"O" ANTIGEN FROM 2% STARCH FURTHER SUBCULTURES	
	Smooth "O" antisera							
	Heterologous	Homologous	Heterologous	Homologous	Heterologous	Homologous	Heterologous	Homologous
S. thompson	G	G	G	G	—	++++		
S. potsdam	G	G	g	g	—	++++		
S. cholerae-suis	G	G	g	g	—	+++		
S. onderstepoort	G	G	G	G++	g	g++	—	+++^{3}
S. poona	G	G	G	G	g	g++	—	+++^{5}
S. minnesota	g	g+	—	++++				
S. illinois	g	g++	sg	sg+++	sg	sg+++	—	++++^{3}
S. newington	G	G	g	g+++	sg	sg+++	—	++++^{4}
S. aberdeen	g	g	—	+++				
S. carrau	G	G	sg	sg+	—	+++		
S. madelia	G	G	g	g+	—	++++		

G = heavy nonspecific granulation; g = slight nonspecific granulation; sg = very slight nonspecific granulation; — = smooth, no granulation, no agglutination; +, ++, +++, ++++ = amount of specific agglutination; numerals indicate the number of further subcultures.

Similar tests were conducted using dextrin or glycogen in place of starch. The results obtained with glycogen were equally good as those obtained with starch, but dextrin was not so satisfactory. Fermentation tests showed that dextrin was fermented by some *Salmonella* strains in 24 hours, and that starch gave a slight acid reaction after 3 to 4 days. Glycogen showed no fermentation.

Since it was noted by White (1929) that the difference in the behavior of smooth and rough cultures toward Millon's reagent was pronounced, a culture of *S. paratyphi* A, which had become smooth after subculturing on starch agar, was tested with this reagent in comparison with two rough strains and a normal smooth strain of *S. paratyphi* B. The smooth strain of *S. paratyphi* A from starch agar and the normal smooth strain of *S. paratyphi* B were negative to Millon's reagent, but the rough strain gave a definite positive reaction.

SUMMARY

When the pH of veal infusion agar did not fall below 7.5 to 7.6 after sterilization, "O" antigens which were grown on this medium agglutinated more heavily in their homologous antiserum. Nonspecific granulation of rough strains was slightly reduced, and agglutination was just visible with smooth homologous antiserum.

Rough cultures which were grown on veal infusion agar, to which had been added 2 per cent starch, became smooth after one or more serial subcultures. From these it was possible to prepare smooth "O" antigens which agglutinated well in their homologous antisera and did not show any nonspecific granulation.

These cultures did not always retain the smooth form when stored in the cold room.

Smooth cultures, whether from starch subcultures or normal smooth strains, all gave a negative reaction to Millon's reagent, whereas rough cultures were positive.

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A METHOD FOR THE ASEPTIC HANDLING OF HIGHLY VISCOUS MATERIALS

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The bacteriological examination of heavy syrups is greatly facilitated by the utilization of a sampling device and a diluent (distilled water), each of which is separately sterilized in individual containers.

It was desired to make bacteriological examinations of malt syrup preparations. The samples, as received from the production tanks, were highly viscous and contained between 60 and 80 per cent solids. Difficulty had been encountered in transferring weighed samples of this material to sterile dilution bottles for agar plating without air contamination. The viscosity of the material precluded pouring the material directly or pipetting.

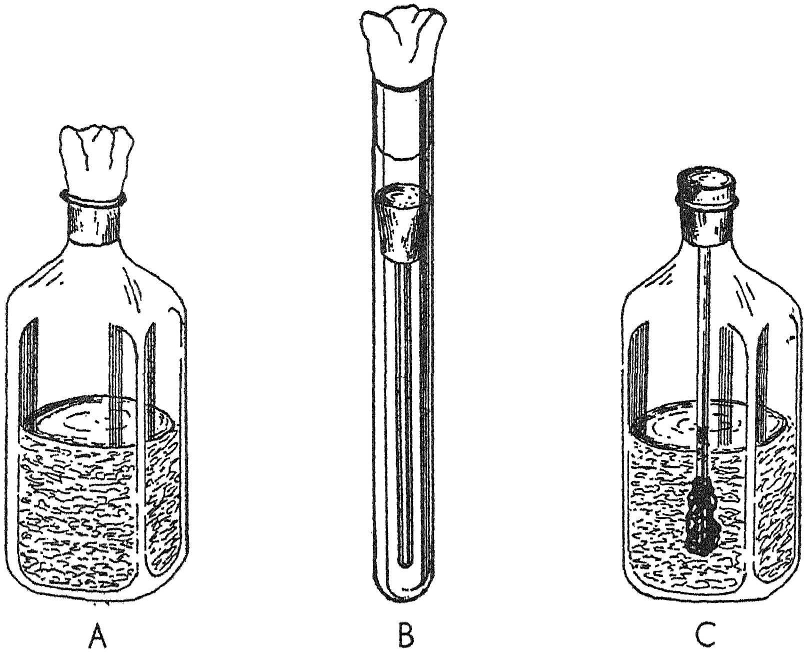

FIG. 1. ASEPTIC HANDLING OF HIGHLY VISCOUS MATERIALS

The following method of handling has proved practicable and convenient. Water dilution bottles containing 99 ml distilled water (figure 1A) are sterilized in the autoclave (15 pounds' pressure, 20 minutes). A rubber stopper (no. 2) is fitted with a glass rod of approximately 4-inch length. The diameter of the glass rod is such as to make an air-tight seal between the rod and the stopper. (The particular diameter of the rod may be varied according to the individual desire and need.) The unit (consisting of rubber stopper and glass rod) is sterilized in a large test tube with a cotton plug (figure 1B). Before use, the rubber stopper and glass rod are inserted into a sterile dilution bottle, aseptic precautions being observed. This assembly, dilution bottle plus rubber stopper and glass rod, is then weighed. A sample of the syrup is then taken by inserting the glass rod (the rubber stopper serving as a handle) into the syrup and twirling the glass rod. The syrup adhering to the rod is transferred to the dilution bottle. With but little practice, an adeptness is developed which permits rapid procurement of the sample on the end of the rod. The bottle plus stopper, glass rod, and syrup (figure 1C) are reweighed, and the sample weight is calculated by difference. Computation of the dilution factor is made directly. A homogeneous mixture is obtained by shaking, and all subsequent handling is performed in a routine manner.

In this laboratory, a triple beam balance has proved to be adequate for most purposes, and, with practice, samples may be routinely obtained having a weight ± 10 per cent from a desired average.

BACTERIAL LYSIS BY LYSOZYME[1]

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The earlier literature on lysozyme sensitivity of bacteria has been extensively reviewed by Thompson (1940). This paper includes a more complete description of the widely used test organism, *Micrococcus lysodeikticus* (Fleming), and further studies on factors influencing bacterial lysis by lysozyme with particular reference to the immunological behavior of the lysozyme substrate.

DESCRIPTION OF THE ORGANISM

Observations were made on cultures obtained from three different sources: one (C) maintained for many years in this laboratory, another (4698) received from the American Type Culture Collection, and a third (F) received recently from Dr. Fleming. To our knowledge all derive from the original organism isolated by Fleming (1922). For purposes of convenience each will be designated as a separate "strain." All three, with a few minor variations, exhibited the same cultural characteristics and could not be distinguished on the basis of the usual laboratory tests. Immunologically, however, there was a significant difference between the C and 4698 strains on the one hand, and the F on the other.

The organism, a gram-positive micrococcus, grows well on ordinary media within a range of pH 7.0 to 7.6 and at an optimum temperature of 37 C. The colonies in undifferentiated cultures are characteristically yellow, convex, opaque, smooth, and glistening. The F strain when received produced a definitely lighter pigment than the other two, and this difference is still apparent after repeated transfer. In broth all cultures usually remain clear, with a sediment which becomes more and more viscid on aging. Better growth is obtained in shallow layers or with aeration. In agar shakes growth occurs on or very near the surface. Reactions in the usual biochemical tests are generally negative. Gelatin is not liquefied. Nitrates are not reduced. Litmus milk becomes slightly alkaline with no other changes. Glucose, lactose, maltose, saccharose, mannitol, and salicin are not fermented. Indole and H_2S are not produced. Starch is not hydrolyzed.

The 4698 strain was used in experiments to develop a synthetic medium which would support the growth of *M. lysodeikticus*. A basal medium containing vitamin-free acid hydrolyzate of casein (General Biochemicals), 0.5 per cent; cystine, 0.01 per cent; tryptophane, 0.01 per cent; salts A and B (according to Snell and Wright, 1941); and adenine, 5 ppm, resulted in visible but poor growth. None of the following substances when added to the basal medium proved to have a stimulatory effect: thiamine, niacin, pyridoxine, pantothenic acid, riboflavin,

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para-aminobenzoic acid, biotin,[2] choline, uracil, pimelic acid, or strepogenin concentrate.[3] The addition of *Lactobacillus casei* factor,[4] 2.5 ppm, however, definitely enhanced growth, and the further addition of 0.5 per cent glucose before autoclaving gave somewhat better results. A typical experiment is shown in table 1. At least 11 serial transfers were possible in this medium. In preparation, the pH of the basal medium was adjusted to 7.4 to 7.6, the accessory substances added, and 5.0-ml amounts were dispensed in 25-ml Erlenmeyer flasks and autoclaved for 15 minutes at 15 pounds. The pH after sterilization was 7.2 to 7.4. The inoculum was 1 drop (0.05 ml by pipette) of a faintly turbid saline suspension of a thrice-washed 18- to 24-hour broth-grown culture. The flasks were incubated for 4 days at 37 C, and the amount of growth was determined turbidimetrically with a spectrophotometer. At the end of the incubation period the pH of the medium was virtually unchanged in the presence of the growing organisms. Since the medium was slightly turbid to begin with, 0.5 ml of 0.1 N HCl was added to each flask to clear the medium before readings were made.

TABLE 1

Growth of Micrococcus lysodeikticus (4698) in a synthetic medium

	AVERAGE OF DUPLICATE READINGS AFTER 4 DAYS' INCUBATION AT 37 C
Basal	76
Basal plus glucose	76
Basal plus *L. casei* factor	57
Basal plus *L. casei* factor plus glucose	47
Blank	100

The percentage transmission was read in a Coleman universal spectrophotometer using the uninoculated medium as a standard transparency of 100. A wave length of 580 millimicrons was used.

This medium also supported the growth of the C and F strains, but it is not known whether the requirements of all three strains are identical. The maximum growth obtained after 4 days' incubation in the medium described above was distinctly poorer than a 48-hour growth in ordinary broth. Whether other accessory substances would further stimulate growth in the presence of the *L. casei* factor remains to be determined. It should be noted that the *L. casei* factor used in these experiments was a "fermentation product material." In two experiments in which a synthetic *L. casei* factor, "folvite," was used, no stimulation was observed. An optimum synthetic medium for *M. lysodeikticus* is still to be defined.

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[2] Biotin was obtained from Merck and Company through the courtesy of Dr. D. F. Robertson.

[3] Strepogenin concentrate was obtained from Dr. D. W. Woolley of the Rockefeller Institute.

[4] The *L. casei* factor used, a "fermentation product material," and also a synthetic preparation, "folvite," were obtained from Lederle Laboratories through the courtesy of Dr. E. L. R. Stokstad.

Variation in colonial morphology has been observed. On buffered neopeptone (Difco) beef infusion agar two types were usually seen: one smooth and opaque, of butyrous consistency and easily suspended; the other much more viscid and more translucent, often adherent to the medium. In some cultures the major difference was in the degree of viscidity and the ease of emulsification of the colonies, rather than in the degree of opacity. The organisms from translucent viscid colonies stained very irregularly with many large, pale, ill-defined gram-negative forms present, in contrast to the regular, evenly stained masses of cocci usually seen. This type of dissociation was more clearly manifest in the C and 4698 strains. Similar colonies were observed with the F strain but were less stable.

TABLE 2

Agglutination tests with Micrococcus lysodeikticus

ANTISERUM	STRAIN	SERUM DILUTIONS						
		1:10	1:20	1:40	1:80	1:160	1:320	1:640
Anti-C	C	++++	++++	+++	+++	+	+	–
	4698	++++	++++	+++	+++	+	+	–
	F	++	–	–	–	–	–	–
Anti-4698	C	+++	+++	+++	+++	+	–	–
	4698	++++	++++	++++	+++	+	–	–
	F	–	–	–	–	–	–	–
Anti-F	C	++	++	++	++	+	–	–
	4698	++	++	++	++	+	–	–
	F	++++	++++	++++	+++	+	+	–

Stable chromogenic mutants from the C and 4698 strains were not infrequently encountered. We have isolated white, pink, and apricot-colored strains. Although extensive studies on the dissociative behavior of these mutants have not been made, it is probable that pigment production varies independently of other characteristics of colonial morphology, as has been described for other organisms (Dubos, 1945).

Although the three strains of *M. lysodeikticus* studied could not be differentiated on the basis of the observations made above, they could be distinguished on the basis of serological tests. Rabbits were immunized with dissociated cultures grown on neopeptone infusion agar to maintain maximum differentiation between smooth and viscid variants. The sera were obtained after 14 intravenous injections of once-washed saline suspensions of living organisms. Agglutination tests were performed in the usual way with organisms grown on either neopeptone or Difco tryptose heart infusion agar. The use of the latter was preferable since on the buffered neopeptone agar the organisms were very difficult to wash off and showed a tendency to autolyze in suspension. This was particularly true of the very viscid variants. Within any one strain no immunological differences could be detected between the smooth and viscid variants by agglutination tests. The C and 4698 strains were always immunologically indistinguishable, but antisera to these strains failed to cross-agglutinate with the F strain beyond a titer of 1:10 or 1:20. Anti-F sera, on the other hand, cross-agglutinated with strains C and 4698 at much higher serum dilutions (table 2). The results of agglutinin absorption tests further emphasized the difference between the C and 4698 strains, and the F strain. It was found that the F strain removed agglutinins for all strains not only from its homologous serum but to a marked degree from anti-C and anti-4698 sera as well, whereas the C and 4698 strains were not capable of removing F agglutinins from F serum. These findings indicate that the American strains, although derived from the same original source, are now distinct from the Fleming strain. Although antisera were not prepared against the chromogenic mutants, suspensions of the latter reacted serologically in the same way as the yellow parent cultures from which they were derived.

SENSITIVITY TO LYSOZYME

Sensitivity to lysozyme was determined by a method previously briefly described (Meyer, 1944). An acid pH is optimum for lysozyme activity; visible lysis of susceptible organisms occurs at an alkaline pH. The organisms from 24- to 48-hour agar slants were suspended in M/15 KH_2PO_4 (pH 4.5) to match the density of a no. 10 $BaSO_4$ standard. To 0.5-ml amounts of serial 2-fold dilutions of pure egg-white lysozyme, in the same buffer, 0.5 ml of suspension were added. The tubes were incubated for 1 hour at 37 C, and, after the addition of 2 drops of N NaOH, were read for clearing of the suspensions. Lysozyme sensitivity was expressed as the highest dilution at which complete lysis of the organism occurred. Within the limits of error of the method, no difference in sensitivity could be detected among the three strains of *M. lysodeikticus* and their variants in repeated tests. As a rule, cultures when tested under the same conditions showed complete lysis at lysozyme dilutions of 1:320,000 to 1:640,000.

Although this method is convenient for the determination of relative sensitivity to lysozyme among different organisms, it is inadequately sensitive for titration and standardization of lysozyme activity. In some instances, lysozyme titers have remained constant over long periods of time in repeated tests with living cultures; in others, unexplainable gross variations in sensitivity occurred from time to time. The usual dilution end point of lysozyme for *M. lysodeikticus* has been between 1:320,000 and 1:640,000, but occasionally the titer has dropped to 1:80,000 or has risen as high as 1:1,280,000 with the same preparation of lysozyme, even when the organism was maintained on the same medium under the same conditions. It may be mentioned here that modifications in the composition of the medium (including changes in pH and the addition of various peptones, sugars, amino acids, etc.), in the temperature of incubation, and in the period of incubation prior to testing, though not always optimum for growth, do not always affect sensitivity of living organisms to a significant degree.

Altered sensitivity could be induced by certain methods of pretreatment of the organisms. Little or no effect resulted from repeated washings with saline, lyophilization, precipitation with ice-cold acetone, treatment with 1 per cent phenol, dialyzation against 0.1 N HCl, or exposure to ultraviolet radiation. Precipitation with either 95 per cent ethyl alcohol or acid acetone, or autoclaving in alkaline solution, rendered the organisms highly resistant to the action of lysozyme. By contrast, organisms autoclaved in acid solution, or formalinized, were almost completely lysed with high dilutions of the enzyme, although it was impossible to obtain complete clearing even with the strongest concentrations used.

In the actual performance of the test numerous attempts were made to enhance the sensitivity by the addition of various reagents. The majority of substances tested had no effect. Ferrous sulfate apparently inhibited the reaction, although the test was difficult to read because of the formation of precipitated ferric hydroxide. Sodium pyrophosphate counteracted this inhibition. Tyrothricin, tryptophane, and the supernatant washings of *M. lysodeikticus* agar slant cultures occasionally seemed to increase the titer, but the results were extremely variable. Of all substances tested, only sodium arsenite was found to have a distinct and consistent effect. With a final concentration of M/200 sodium arsenite, the titer was 4 times higher than that of controls without arsenite.

Isolation from *M. lysodeikticus* of the high polymer form of the lysozyme substrate has recently been accomplished. This mucopolysaccharide substrate is rapidly depolymerized by lysozyme, as indicated by viscosity tests. The method of isolation and characterization of the substrate and the technique of the test are described in detail elsewhere (Meyer and Hahnel, 1946). Optimum conditions for the antibacterial action of lysozyme parallel in general the conditions for its depolymerizing action. The latter test, however, is specific, accurate, and rapid, and is not encumbered by the uncontrollable biological variations which seem to be inherent in tests with the intact organism. A comparison between the bacteriological and chemical tests, not only with regard to egg-white lysozyme but to lysozymes of plant origin as well, is likewise described elsewhere (Meyer, Hahnel, and Steinberg, 1946). For the accurate determination and standardization of lysozyme activity the bacteriological test has been supplanted by a chemical test which is constant and reproducible.

An interesting observation was made with regard to the behavior of an adapted strain of *M. lysodeikticus* which became resistant to egg-white lysozyme. This strain required 16 times as much egg-white lysozyme for complete lysis as the parent C strain from which it was derived and became completely refractory to ficus lysozyme (Meyer, Hahnel, and Steinberg, 1946). Only one-tenth the amount of carbohydrate substrate yielded by the parent strain could be extracted from the adapted strain. Yet the nature of this substrate was apparently unchanged since it was hydrolyzed to the same degree by both egg-white and ficus lysozyme. The complete resistance to ficus lysozyme of the intact adapted organisms could not be explained by the ability of the latter to destroy this enzyme. Supernatants from ficus lysozyme incubated with the adapted strain revealed no apparent destruction of the enzyme when tested against the susceptible parent strain. Adapted organisms killed by treatment with phenolized saline were also completely refractory. The most probable explanation for the complete resistance of these organisms to ficus lysozyme would seem to be that the polarity of the substrate complex as it exists on the bacterial surface is changed so that it can still combine with the strongly electro-positive egg-white lysozyme but not with the more electro-negative ficus lysozyme.

IMMUNOLOGICAL BEHAVIOR OF THE SUBSTRATE

The lysozyme substrate used in these experiments was obtained from the C strain of *M. lysodeikticus* by fractionated extraction with 0.5 N NaOH and fractionation with alcohol after the removal of proteins and other impurities. This substance is a high polymer mucopolysaccharide fraction which in its present crude state of isolation contains about 5 per cent nitrogen and about 30 per cent hexosamine. It is rapidly depolymerized by lysozyme and hydrolyzed, with the liberation of reducing sugar, half of which can be accounted for as acetylglucosamine. For immunization and precipitin tests the mucopolysaccharide was dissolved in saline. It proved to be antigenic, although weakly so, when tested in a single rabbit. After subcutaneous, intraperitoneal, and intravenous injections of a total of 56.0 mg, the serum obtained gave a very weak precipitin reaction with the substrate (1.0 ml precipitated with 5 to 10 μg of substrate after standing for several days in the icebox) and a very low agglutinin titer against the homologous organism. By contrast, 3 of 4 rabbits[5] immunized with living organisms produced much stronger precipitating antibodies against the substrate, in addition to good agglutinin titers, after 14 intravenous injections; and after a further series of 16 injections, 0.5 ml of all these sera precipitated almost immediately with 10 to 100 μg of substrate.

It was found that a saline solution of substrate in a concentration of 1.0 mg per ml still gave a precipitin reaction after standing in the icebox for 3 weeks. Apparently any changes in viscosity that may have occurred did not affect the combining properties of the substrate. This was borne out by the following experiment in which depolymerized substrate was used. The substrate was prepared for use as in a viscosity test (Meyer and Hahnel, 1946), and, following the addition of lysozyme, samples were withdrawn every 10 minutes over a period of 1 hour. Each sample was diluted in saline to contain 50 μg of substrate per 0.5 ml and added to 0.5 ml of a 1:10 dilution of a strong antiserum. The relative viscosity was 3.6 initially and fell to 1.6 in an hour. All the samples tested as depolymerization progressed showed immediate turbidity and the same amount of precipitate as far as could be judged visually by this kind of a test. The degree of hydrolysis as determined by an increase in reducing sugar was negligible under the conditions of this experiment.

Attempts were made to determine the relationship of the substrate-precipitating antibodies to the agglutinating antibodies. Precipitin tests were set up in which varying amounts of substrate antigen were added to constant amounts of antiserum. With the antibacterial sera used in these experiments, turbidity, followed by precipitation, was immediately apparent. The tubes were placed in the icebox for 48 hours and the amount of precipitate formed noted by visual inspection. After centrifugation, the supernatants were withdrawn from each tube and divided into two portions, one of which was tested for excess antibody by the addition of substrate antigen, the other for excess antigen by the addition of antibody. The results of a typical experiment, as given in the left half of table 3, show that there was a considerable range in which both antigen and antibody were present in the same supernatant. Such behavior may be due to the presence of several antigens in the substrate or to some alteration in the process of isolation (Kendall, 1937). When similar supernatants were tested for the presence of agglutinating antibodies against the organism, the titer remained virtually undiminished as far as could be determined by the usual crude agglutination tests. Absorption of antibacterial serum by the organism, on the other hand, removed both precipitating and agglutinating antibodies. Lysozyme substrate thus appears to be one of the antigens of *M. lysodeikticus*, presumably forming part of

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[5] Two of these were anti-C and 2 were anti-4698; no differences in their behavior against C substrate were noted.

TABLE 3

Addition of increasing amounts of substrate to 1.0 ml anti-M. lysodeikticus serum no. 367 diluted 1:10

TUBE*	SUBSTRATE ADDED µg	REACTION†	TESTS ON SUPERNATANTS		EFFECT OF ADDITION OF SUBSTRATE‖ TO SUPERNATANTS FROM TREATED PRECIPITATES			
			Excess antigen‡	Excess antibody§	Lysozyme-treated		Controls	
					Immediate	24 hours	Immediate	24 hours
1	150	+	++++	+				
2	100	+	++++	+	t	++++	–	–
3	80	+	++++	+	t	+++	–	–
4	60	++	++++	+	t	+++	–	v sl t
5	50	+++	++++	+	t	+++	–	t
6	40	+++	++	+	t	+++	–	sl t
7	30	++++	+	+	t	+	–	sl t
8	20	++++	t	+	t	+	–	sl t
9	10	++++	t	++	t	+	–	sl t
10	8	++++	–	++++				
11	5	+++	–	++++				
12	3	+++	–	++++				
13	1	+	–	++++				

* Set up in duplicate; precipitates used for action of lysozyme on precipitated substrate.

† Read after 48 hours' incubation in the cold: to ++++ indicates relative amount of precipitate; t, turbid; v sl t, very slightly turbid.

‡ Tested by addition of 0.5 ml 1:10 dilution of serum.

§ Tested by addition of 2.5 µg substrate to tubes 1 through 7, 5.0 µg to tube 8, 10 µg to tubes 9 and 10, and 100 µg to 11, 12, and 13.

‖ 20 µg substrate added.

the cell surface. The antibody which precipitates the isolated substrate seems to be one of the agglutinins produced by injection of the whole organism.

With regard to the immunological specificity of the substrate little can be said until more data are available. Substrates were prepared from the F strain of *M. lysodeikticus*, and from two other lysozyme-sensitive organisms, *Sarcina lutea* and *Staphylococcus muscae*.[6] Two rabbits had been immunized with the F strain and two with *S. lutea*, using saline suspensions of living organisms. All of these sera had only very weak precipitating antibodies against their homologous substrates, and against the C substrate as well. The anti-C and anti-4698 sera precipitated the F and *S. lutea* substrates to the same degree as the C substrate when observations were made using 0.5-ml amounts of serum and 10 and 50 μg of substrate. The *S. muscae* substrate was not precipitated by any of these sera. These observations warrant the conclusion that the *M. lysodeikticus* and *S. lutea* substrates are closely related immunologically. Until specific sera with good titers can be prepared against all the individual substrates, it will be difficult to determine their exact antigenic relationships. Elucidation of the relationship of these substances to one another and to other antigens in the bacterial cell may require the application of quantitative immunochemical methods, using more highly purified substrates.

Experiments were set up to determine whether whole organisms and the substrate in combination with antibody were still susceptible to the action of lysozyme. The initial observations were made previously on agglutinated organisms. In these experiments, the technique for determining sensitivity was the same as described above, except that saline was used both for suspending the organisms and for dissolving the enzyme. At this pH (6.8 to 7.0) gradual lysis could be observed without the addition of NaOH, although the latter was added before final readings were made. It was found that a suspension of agglutinated *M. lysodeikticus* showed the same sensitivity to lysozyme as a control suspension in normal rabbit serum, i.e., complete lysis at 1:640,000 dilution of lysozyme. In a further test, a constant amount of the enzyme was added to a series of tubes containing organisms agglutinated by serial 2-fold dilutions of antiserum. For this purpose an agglutination test was first set up in the usual way with a serum dilution range of 1:10 to 1:640. After incubation at 37 C for 1 hour and then in the icebox overnight, 10 μg of lysozyme were added to each tube, including an unagglutinated control suspension, and after 1 hour at 37 C the tubes were read for lysis. Lysis of agglutinated cocci always occurred, even in the presence of the highest concentrations of serum, i.e., 1:10, in which the supernatants still showed the presence of excess agglutinating antibodies. This was true for all strains of *M. lysodeikticus* and for *S. lutea* as well.

The ability of lysozyme to attack a precipitated substrate was then studied in the following way: The precipitates formed in the experiment outlined in table 3 were allowed to stand in the cold for 48 hours and then centrifuged in the cold.

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[6] The *S. lutea* used in our experiments is as sensitive to lysozyme as *M. lysodeikticus*, lysis occurring at 1:320,000 to 1:640,000 dilutions of lysozyme. *S. muscae* is much less sensitive, lysis occurring at 1:10,000 to 1:20,000 dilutions.

The supernatants were decanted and drained off, and the precipitates resuspended in chilled saline and again centrifuged in the cold. The supernatants were again discarded, the precipitates suspended in 1.0 ml M/15 Sorensen PO_4 buffer, pH 5.0, and 100 μg of lysozyme added in 0.1 ml of the same buffer. For each series of tubes a similar control series was set up without the addition of enzyme. The treated precipitates and controls were incubated for 20 to 24 hours.[7] At the end of this time an unmistakable difference in the appearance between the treated and untreated precipitates was noted. In the controls the precipitates remained as opaque white pellets, whereas in the treated series they appeared as translucent vacuolated material closely adherent to the bottom of the tube and markedly diminished in size. It could be shown that some antibody had been released as a result of lysozyme action.[8] The supernatants were withdrawn from all tubes and the pH adjusted to 7.2. Twenty μg of substrate were added and the immediate reaction was noted. In the supernatants from treated precipitates the tubes became turbid immediately and precipitation was observed on the following day. The control series by comparison remained clear or became very slightly turbid. The findings are summarized in the right half of table 3. The evidence indicates that lysozyme is still capable of attacking organisms or substrate when either is combined with antibody.

The assumption that the substrate occurs as a surface antigen in the bacterial cell and may be covered by antibody during agglutination seems reasonable in view of the fact that the intact organism removes antisubstrate as well as agglutinating antibody. If this assumption is valid, the fact that agglutinated bacteria are still susceptible to lysozyme lysis suggests that with the intact organism, as with the isolated substrate, the enzyme is capable of penetrating the antigen-antibody complex to attack the substrate. It has been demonstrated (Delbrück, 1945) that high concentrations of antibacterial antibody will inhibit the adsorption of bacteriophage onto the bacterial cell. This difference in behavior between a virus and an enzyme may possibly be explained by the difference in size between the two agents.

SUMMARY

Cultures of *Micrococcus lysodeikticus* obtained from three different sources were identical in their cultural characteristics and could be grown in a semisynthetic medium. On the basis of immunological tests, two of these strains were found to be identical, but the third differed significantly from the other two.

All cultures of the organism and of its variants and chromogenic mutants showed the same sensitivity to egg-white lysozyme when tested under the same

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[7] Under these conditions hydrolysis of the substrate can be expected to occur, since it is customary to add 200 μg of lysozyme to 5.0 mg of substrate and test for hydrolysis at the end of 2 hours.

[8] This is not to imply that the precipitates in the control series would not dissociate spontaneously to some extent under the described experimental conditions. Also it should be remembered that some lysozyme is probably still present in the supernatant from the treated precipitates. For this reason the immediate reaction was noted before the enzyme could conceivably hydrolyze the newly added substrate.

conditions. An adapted strain which showed a marked decrease in sensitivity to egg-white lysozyme became completely refractory to ficus lysozyme.

The high polymer mucopolysaccharide lysozyme substrate isolated from *M. lysodeikticus* proved to be weakly antigenic when tested in a single rabbit, giving rise to substrate-precipitating antibodies as well as agglutinins. It was precipitated by antibacterial serum and is one of the antigens of the organism. The substrates of *M. lysodeikticus* and *Sarcina lutea* are closely related immunologically. Depolymerized substrate is still capable of combining with antibody.

Lysozyme is capable of attacking organism or substrate when either is combined with antibody.

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THE FRAGMENTATION OF THE MYCELIUM OF PENICILLIUM NOTATUM AND PENICILLIUM CHRYSOGENUM BY A HIGH-SPEED BLENDER AND THE EVALUATION OF BLENDED SEED

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Penicillin is currently being produced almost entirely by a submerged fermentation for reasons already stated by Moyer and Coghill (1946). For this fermentation, a reduction in the volume of seed and a reduction in the number of steps in production would have the advantage of simplifying the preparation of seed and minimizing the risk of contamination. Reducing the number of steps would also hold to a minimum any loss of penicillin productivity from seed deterioration (Clutterbuck *et al.*, 1932; Foster *et al.*, 1943). Any concomitant sacrifice in yield or delay in the final fermentation cycle would, of course, be undesirable. This is a report of experiments, which have been conducted on a small scale, testing a method of preparing seed which may find application in larger scale production.

Most fungi, including the penicillin-producing penicillia, produce both mycelium and spores. Fermentations brought about by these fungi may vary in their course and extent with the quantity and type of seed unit employed, a seed unit being any viable spore, spore cluster, hypha, or hyphal cluster. Certain members of the *Penicillium notatum-chrysogenum* group produce such large mycelial clusters that they are visible as discrete pellets, 1 to 5 mm in diameter. Since hand shaking of a flask of these pellets causes no fragmentation, we tried various mechanical methods in an effort to induce separation of the mycelium at its cross walls. Of the methods tried, blending in a high-speed propeller type blender (Waring "blendor") proved to be most satisfactory, and throughout this report we shall refer to vegetative seed so fragmented as "blended" seed.

We have not been interested solely in measuring the increase in seed units brought about by blending, for mere growth of fragments would be no guarantee of efficiency in penicillin production. Rather, we have been interested in determining how small an amount of blended seed will substitute adequately for 10 per cent seeding with unblended vegetative pellets, which serve as a control.[1]

Two strains of penicillia, *Penicillium notatum*, NRRL 832, and *Penicillium chrysogenum*, NRRL 1951, both obtained from the Northern Regional Research Laboratories at Peoria, Illinois, have been submitted to parallel treatment. To eliminate the factor of strain variation during the study, we used spores from two large reserves of aqueous spore suspensions, stored at 4 C, for the preparation of vegetative seed as needed. The suspension of NRRL 832 spores contained 0.5×10^9 spores per ml; that of NRRL 1951 spores contained $2.0 \times$

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[1] Ten per cent vegetative seed was considered essential at the time these studies were started.

10^9 scores per ml. These suspensions were kindly made and counted for us by Dr. A. J. Whiffen of these laboratories.

EXPERIMENTAL PROCEDURE

Erlenmeyer flasks of 500-ml capacity were used routinely as "shaker flask" fermenters. Each flask contained 100 ml of sterile corn steep medium, prepared according to the following formula:[2]

Lactose	20.0 g
Corn steep liquor (50 per cent solids)	40.0 ml
$MgSO_4$	0.125 g
KH_2PO_4	0.250 g
$NaNO_3$	1.00 g
Tap water to	1,000 ml
pH, after sterilization, 4.5	

Seeding was accomplished with measured volumes of spore or mycelial suspensions. Triplicate flasks were seeded at each level, and the fermentation was conducted at 24 C in a shaking machine of the oscillating type. The length of stroke was 4 inches; there were 95 such complete oscillations per minute.

Samples were taken daily, starting usually on the third day of the cycle. A minimal amount, about 3.5 ml, was removed from each of the three replicate flasks to give a pooled sample for that level. This gave a sufficiently large sample for assay and determination of pH, without materially diminishing the beer volume in each flask.

All pooled samples were allowed to stand for 10 to 15 minutes. One-ml volumes of the clear, supernatant beer were then transferred to sterile vials and submitted for assay of penicillin content by a hollow-cup agar-plate method. This method, as described in Circular 198 of the Department of Agriculture (1931), was adapted to the assay of penicillin by Dr. J. F. Norton of these laboratories. Each assay value was determined by averaging four zones of unknown from a single plate and translating this average diameter into Oxford units per ml from the standard curve prepared for that day.

TITRATION OF OPTIMAL SPORE SEED LEVEL

Spore seeding was performed at various levels in order to establish the optimal rate of seeding. In figures 1 and 2 are shown the influence of rate of spore seeding upon the fermentation cycle. The curve for each level of spore inoculum is "set over" five days from the curve for the preceding level, to avoid the confusion of too many overlapping curves.

From figures 1 and 2 it may be seen that too small or too large an inoculum is not productive of maximal potency levels. Seeding rates between 1,000 and

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[2] This formula was communicated to us by Dr. A. J. Moyer of the Northern Regional Research Laboratories in a copy of one of the restricted reports to Dr. A. N. Richards, chairman, Committee on Medical Research, Office of Scientific Research and Development. Its composition is given here since it is not included in the recent publication of Moyer and Coghill (1946).

1,000,000 spores per ml caused the fermentation to reach maximal potencies in almost identical lengths of time. Strain NRRL 832 was capable of producing penicillin levels of approximately 80 u (Oxford units) per ml and strain NRRL 1951 was able to produce about 100 u per ml at optimal spore seed levels.

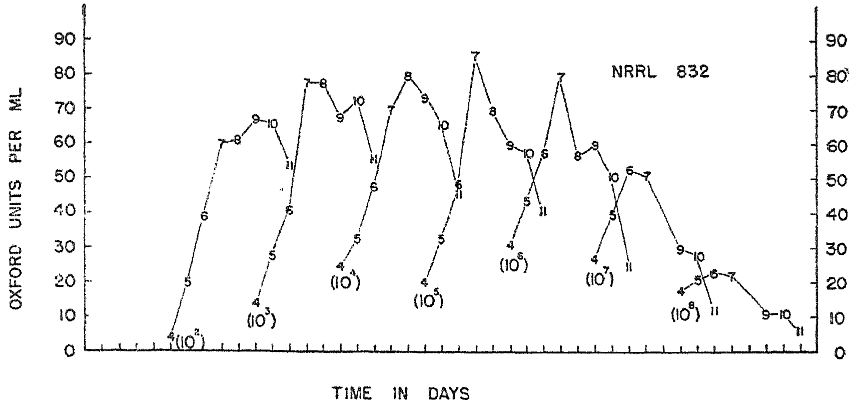

FIG. 1. PENICILLIN FERMENTATION CURVES SHOWING THE INFLUENCE OF RATE OF SPORE SEEDING FOR P. NOTATUM, NRRL 832

Numbers in parentheses refer to the number of spores per ml of substrate to give the corresponding curve. Numbers are used as data points to indicate the day of the fermentation cycle.

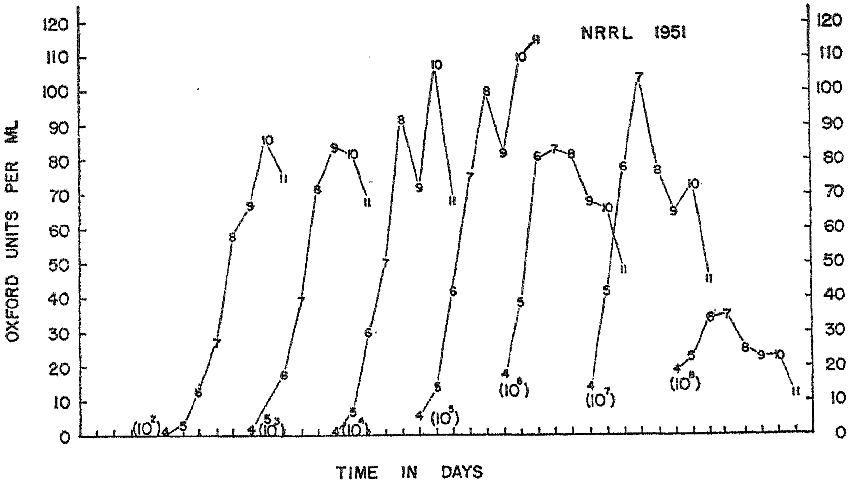

FIG. 2. SAME AS FIGURE 1, BUT FOR P. CHRYSOGENUM, NRRL 1951

STUDY OF BLENDING TIME

Mycelial growth of these two fungi in a shaker flask reaches a maximum in from 3 to 6 days. At this time most of the liquid volume is occupied by pellets of mycelium. One flaskful of this seed, approximately 100 ml, is a convenient volume to blend at one time in the usual 1-quart-size blender jar.

This operation is performed aseptically in a jar equipped with a special type of metal cover and splash plate, as shown in figure 3.

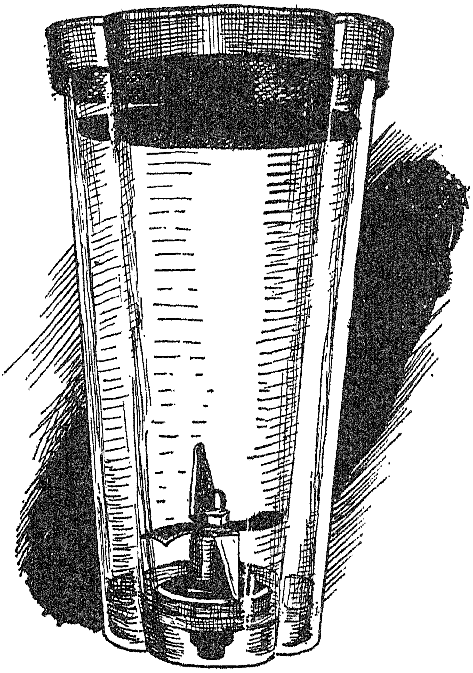

FIG. 3. THE TYPE OF COVER WITH BAFFLE MADE OF SHEET COPPER USED WITH WARING "BLENDOR" JAR TO CARRY OUT STERILE BLENDING OPERATION

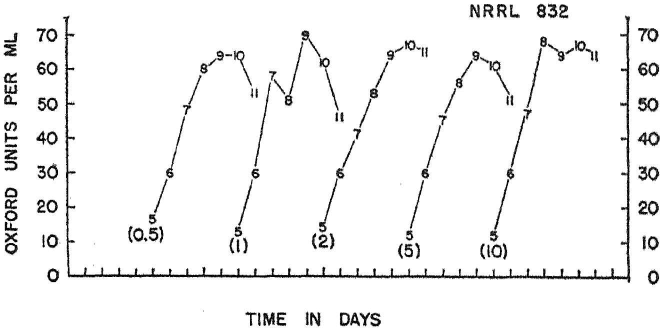

FIG. 4. INFLUENCE OF BLENDING TIME UPON PENICILLIN FERMENTATION CURVE
Seeding rate is constant, at 1:100,000. Culture is *P. notatum*, NRRL 832. Numbers in parentheses refer to minutes of blending time.

During the first half minute of blending, a visible change in the consistency of the slurry occurs. A "titration" of the blended seed for increase in seed units was conducted at the time intervals of 0.5, 1, 2, 5, and 10 minutes. The fermentation curves obtained from seed blended for these various time intervals are shown in figures 4 and 5.

A continuous blending for more than two minutes is detrimental to these fungi, since, during the blending operation, heat develops from the operation of the high-speed (10,000 rpm) blending blade. The seed prepared for the 5- and 10-minute blending-time studies shown in figures 4 and 5 was protected from this heat effect by cooling the blender jar and contents between each blending period of not more than 2 minutes.

From data of figures 4 and 5, we adopted 2 minutes as a desirable length of time for routine blending. In 2 minutes, most of the hyphal fragments contain between 1 and 10 cells, with an average of about 4 cells per fragment. About 20 per cent of the fragments are created by actual fracture of the cell wall. The terminal cells of the rest of the fragments are undamaged, and these fragments appear to have been formed by fragmentation at the septa, under the physical stress of blending.

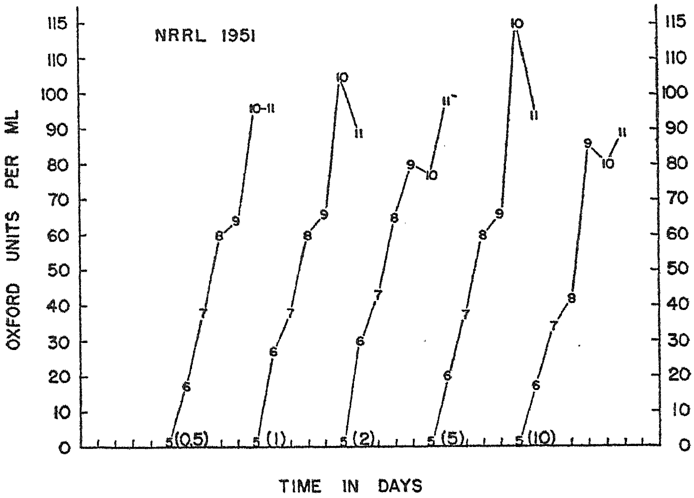

FIG. 5. SAME AS FIGURE 4, BUT FOR P. CHRYSOGENUM, NRRL 1951

TITRATION OF BLENDED SEED

Six-day-old vegetative seed was blended for 2 minutes and diluted in sterile medium so that final dilutions of 1:2,500; 1:10,000; 1:40,000; 1:100,000; 1:250,000; and 1:500,000 could be fermented parallel to 1:10 dilutions of the unblended seed, which served as a control. Data so obtained are shown as solid lines in figures 6 and 7.

Similar results were obtained with 4-day-old seed blended on the second as well as the fourth day, and with 6-day-old seed, blended on the third as well as the sixth day. Twice-blended seed, since it was able to mend between its first and second blending periods, is referred to as "B.M.B." seed. Comparisons of singly blended seed (B.) with doubly blended seed (B.M.B.) are also shown in figures 6 and 7.

Blended seed seems to reach slightly higher peaks about 2 days later than the

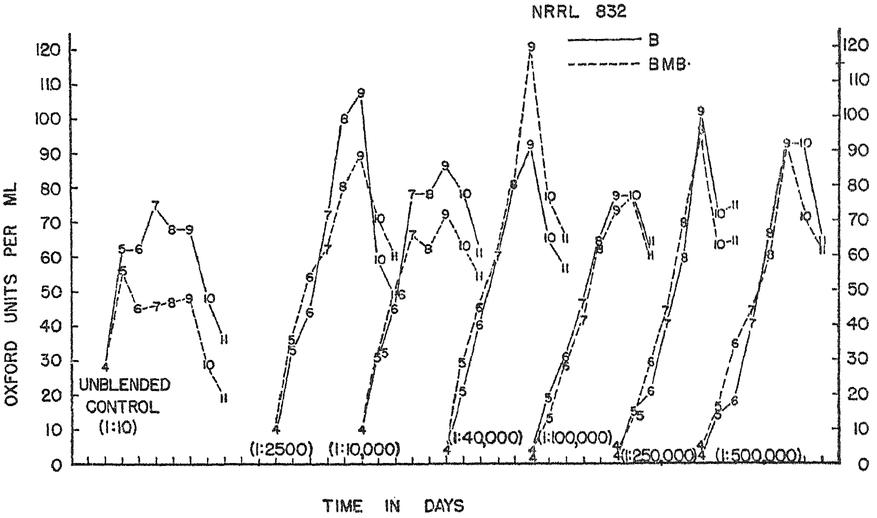

Fig. 6. Titration of Two-Minute Blended Seed Showing the Influence of Dilution upon the Fermentation Curve

Unblended seed was run as a control at a seeding rate of 1:10. Numbers in parentheses refer to the dilutions of blended seed giving the corresponding fermentation curves for *P. notatum*, NRRL 832.

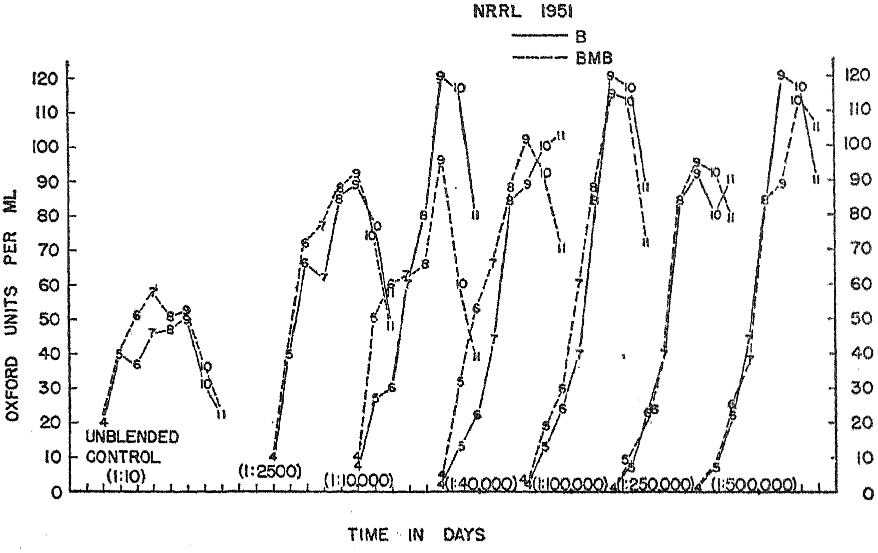

Fig. 7. Same as Figure 6, but for P. chrysogenum, NRRL 1951

controls. At the peak for the controls (seventh day), blended seed, diluted as much as 1:40,000, gives as good production as the controls, and the productions from the 1:100,000, 1:250,000, and 1:500,000 series are almost as good.

DISCUSSION

Some fungi, e.g., the oidia among transitional yeastlike fungi (Henrici, 1930) and *Nocardia* among the actinomycetes (Waksman and Henrici, 1943), tend to fragment automatically or with very little mechanical stress applied. Apparently fragmentation can be mechanically induced in certain fungi whose mycelium resists fragmentation under the small stresses of hand shaking and ordinary routine handling. At any rate, two penicillia have been shown to endure fragmentation by blending, with no apparent disturbance of growth and fermentative properties. This property of withstanding damage under blending operation may allow blended seed to be used as a substitute for much larger volumes of unblended seed.

During the delay in publication of this work, there have been isolated and tested new members of the *P. notatum-chrysogenum* group which give higher yields and which grow as much smaller pellets or clusters of mycelium. It may well be that this type of vegetative seed can be used wisely at such low seeding rates as 1:10,000 or 1:40,000 without blending. The principle of using very small seed volumes, whether the seed is blended or unblended, will have to be evaluated in terms of current practice with large fermenters.

SUMMARY

Two fungi, *Penicillium notatum*, NRRL 832, and *Penicillium chrysogenum*, NRRL 1951, have been shown to endure blending of their vegetative mycelium by a Waring "blendor" with little if any injury to their growth and fermentative capacities. Fragmentation seems to occur usually at the septa.

Such blended seed, when diluted as much as 1:40,000 times, adequately substitutes for unblended seed at a 1:10 seeding rate, in shaker flasks.

Blended, mended, and blended seed is not superior to singly blended seed.

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ISOLATION OF STREPTOMYCIN-PRODUCING STRAINS OF STREPTOMYCES GRISEUS[1,2]

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In the study of the production of antibiotics by microorganisms, especially when a given substance receives recognition as a chemotherapeutic agent, it becomes necessary, in order to maintain the potency of the culture cr to obtain more active cultures, to isolate fresh strains of the organism producing the particular antibiotic. This can usually be accomplished by two procedures: (1) Fresh cultures of the organism are isolated indiscriminately from various natural substrates, such as soils and composts, and tested for their potency. (2) New strains are obtained by plating the original culture and then isolating individual colonies. Before plating, the culture may be pretreated, as by exposure to different radiations, in order to kill a large number of sensitive spores. Both of these methods have been utilized with considerable success in the isolation of more potent strains of penicillin-producing fungi. Comparatively little progress has been made, however, in the case of streptomycin-producing strains of *Streptomyces griseus*.

It has been established that penicillin production is characteristic of the *Penicillium notatum* and *Penicillium chrysogenum* groups; the variation in potency of different strains is either quantitative or qualitative, according to the type of penicillin produced. The production of streptomycin, however, is characteristic of only a certain few strains of *S. griseus* (Waksman, Schatz, and Reynolds, 1946). This organism represents a distinctly heterogeneous group, especially in regard to the production of antibiotics. In a recent examination of 40 freshly isolated cultures of *S. griseus*, none was found to produce the typical streptomycin; only one of these cultures was found to form an interesting antibiotic. This antibiotic was active against certain gram-positive and gram-negative bacteria, in a manner comparable to streptothricin and streptomycin, but it was both chemically and biologically distinct from either of these two substances. These results pointed to the difficulty of obtaining fresh streptomycin-producing cultures from natural substrates. Numerous attempts to isolate streptomycin-producing strains of *S. griseus* from natural substrates have so far yielded, in addition to the two original strains obtained in this laboratory in 1943, namely, D-1 and 18-16 (Schatz, Bugie, and Waksman, 1944), only two cultures. One isolation was made in another laboratory, and the

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[1] Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

[2] With partial support by a fellowship established by Merck and Company of Rahway, New Jersey.

other isolation in our laboratory, as reported here. All the cultures, however, that are now being used in industrial organizations for the production of streptomycin have been isolated from one of our original strains, namely, 18-16 (Schatz and Waksman, 1945).

It was also established that the streptomycin-producing culture of *S. griseus* produces inactive mutants or strains (Schatz and Waksman, 1944). This necessitates continuous purification of the culture and use of the more active variants, not only to increase the yield of streptomycin, but also to prevent the continuous deterioration of the culture.

In order to facilitate the isolation of *S. griseus* from natural substrates, advantage was taken of the following principle: Microorganisms producing certain antibiotics are usually more resistant to the action of these antibiotics than are other organisms not capable of forming such antibiotics. This was found to hold true even of closely related forms, as shown for the inactive mutant of *S. griseus*, which is sensitive to streptomycin. In order to facilitate the isolation of new and more potent strains of antibiotic-producing organisms, the particular antibiotic may be incorporated in the medium in concentrations sufficient to inhibit the growth of other organisms and of inactive strains of the same organisms. This, however, does not affect the growth of potent strains.

TABLE 1

Inhibition of different actinomycetes by their respective antibiotics

ANTIBIOTIC	ORGANISM PRODUCING IT	ACTIVITY OF PREPARATION PER 1 MG	DILUTION UNITS PER MG, EXPRESSED AS ACTIVITY AGAINST		
			S. antibioticus	*S. lavendulae*	*S. griseus*
Actinomycin..........	*S. antibioticus*	100,000*	100	5,000	100
Streptothricin........	*S. lavendulae*	100†	1,000	0.4	10
Streptomycin.........	*S. griseus*	125†	1,000	100	1.2

* *S. lutea* units; crystalline material.

† *E. coli* units; crude preparation.

The principle underlying this method can be illustrated by the selective activity of three antibiotics produced by actinomycetes against the mother culture (table 1). Actinomycin, streptothricin, and streptomycin were selected because of their distinct bacteriostatic spectra and the ease with which differences in antibacterial action can be demonstrated. Their activity was expressed in dilution units per 1 mg of the respective preparations. Actinomycin was measured in terms of *Sarcina lutea* units, since *Escherichia coli* is resistant to it, whereas the potency of the other two antibiotics was expressed as *E. coli* units. Activity was measured by the agar streak method (Waksman and Reilly, 1945). *Streptomyces antibioticus* is fairly resistant to actinomycin, its own antibiotic, but is sensitive to streptothricin and streptomycin. *Streptomyces lavendulae* is also resistant to its own antibiotic, streptothricin, but it is sensitive to streptomycin, an antibiotic related to it, and it is especially sensitive to actinomycin. *S. griseus* is also most resistant to its own antibiotic, streptomycin; it shows a certain degree of resistance to streptothricin, but it is far more sensitive to actinomycin.

On the basis of these results one would be justified in concluding that the addition of streptomycin to nutrient media would tend to eliminate non-streptomycin-producing organisms, namely, various bacteria, as well as the majority of actinomycetes, but not fungi which are resistant to streptomycin. This medium should thus favor the development of organisms which either produce streptomycin or are resistant to it. This medium should also prove favorable, not only to the isolation of fresh cultures from natural substrates, but also to the elimination of inactive strains from a streptomycin-producing culture. The results (table 2) of a comparative study of the sensitivity of different strains of *S. griseus* to streptomycin, on the one hand, and to the living culture of an active streptomycin-producing strain of *S. griseus*, on the other, further serve to illustrate this principle.

TABLE 2

Sensitivity of 8 different strains of S. griseus to streptomycin and to the antagonistic action of S. griseus 3463-4

STRAIN OF *S. griseus* NO.	GROWTH IN PRESENCE OF STREPTOMYCIN (μG/ML) IN NUTRIENT AGAR*			ZONE OF INHIBITION PRODUCED BY NO. 4, MM†
	50	10	1	
3326	0	0	+	25
3378	0	0	+	24
3463	+++	+++	+++	6
3464	+++	+++	+++	5
3463-4	+++	+++	+++	6
3475	+++	+++	+++	6
3478	trace	++	+++	16
3481	+++	+++	+++	7

* 0 = no growth; trace, +, ++, +++ = relative amounts of growth.

† Cross streak tests on nutrient agar.

Eight strains of *S. griseus* were used:

3326—Original type culture of the organism, isolated from soil in 1915 and kept in the collection. Inactive.

3378—Isolated by Dr. M. B. Morrow of Texas, from a soil in Yucatan. Inactive.

3463—Original 18-16 culture which produced streptomycin.

3464—Original D-1 culture which produced streptomycin.

3463-4—Active isolate from 18-16, obtained in our laboratory.

3475—Active isolate (42.1) obtained by Dr. H. W. Anderson of the University of Illinois from our strain 3863-4.

3478—A culture designated as G-25, which produced an antibiotic of the nonstreptomycin type.

3481—A streptomycin-producing culture of *S. griseus* freshly isolated from soil by the streptomycin-enriched medium.

The results show that strains 3326 and 3378, the non-streptomycin-producing strains, are very sensitive to streptomycin. The two original streptomycin-producing cultures (18-16, and D-1) and their isolates (3863-4 and 3475) are very resistant, a fact brought out particularly by the streak method. The freshly isolated culture (3481) is also resistant to streptomycin, but somewhat less so than the others. The sensitivity of G-25 fell between that of the inactive and the streptomycin-producing strains.

In order to demonstrate growth on the streptomycin-enriched medium of streptomycin-producing and non-producing strains found in the same culture, the results of the following experiment may be reported. A suspension of spores of strain 3463-4 was plated out on ordinary nutrient agar as well as on the same agar plus 100 μg of streptomycin per ml. The corresponding numbers of colonies obtained on the two media were 20.4 and 5.8 millions per ml of spore suspension, indicating that nearly 80 per cent of the colonies are sensitive to 100 μg of streptomycin per ml. Ten colonies were picked at random from the nutrient agar plates and grown on glucose-asparagine agar. Six of these colonies produced an atypical, reddish, vegetative growth and a typical, greenish-gray mycelium. The remaining four colonies, as well as all colonies picked from the streptomycin-enriched agar plates, produced the typical growth of *S. griseus*. The atypical colonies produced no streptomycin, as shown in table 3.

TABLE 3

Production of streptomycin by freshly picked colonies from S. griseus plates

(Shaken cultures)

COLONY NO.	ACTIVITY, μG PER ML AFTER INCUBATION OF	
	3 days	6 days
NA 1*.	<5	<5
NA 2.	24	28
NA 4.	<5	<5
NA 6.	28	28
SNA 2†.	14	38
SNA 6.	13	46

* Strains NA 1, 2, 4, and 6 were isolated from nutrient agar plates, 1 and 4 being atypical colonies, since they produced on glucose-asparagine agar slants a red vegetative mycelium.

† Colonies SNA 2 and SNA 6 were isolated from nutrient agar enriched with 100 μg per ml of streptomycin.

In order to establish again whether streptomycin-enriched agar can be used for the isolation of fresh streptomyin-producing cultures, agar media containing 25 to 100 μg of streptomycin per ml were used for plating out various natural materials, such as soil, peat, and compost. These media depressed the development of nearly all the bacteria and actinomycetes, but had virtually no effect upon the growth of the fungi. A few actinomycetes developed on these media, but they grew only very slowly. Many of them proved to belong to the *S. griseus* group. They were isolated and cultivated on media favorable for the production of streptomycin. Some of them produced antibiotic agents. Most of these antibiotics were found, however, to be not of the streptomycin type. Three of the resistant cultures were grown on different media, both in stationary and in submerged culture, and the culture solutions were tested for their activity against several bacteria. Only one showed fairly high activity against *Bacillus subtilis*, when grown on all media, and limited activity against *E. coli* and *Bacillus mycoides*. The properties of this agent did not seem to fit in with those of streptomycin.

One culture (3481) isolated during one of these surveys, however, appeared to be definitely of the streptomycin type. The substrate from which this culture was isolated was a soil adjoining a dairy barn and was thus heavily manured. The culture filtrate gave 30 units of activity against *E. coli*, and 100 against *B. subtilis*, *B. mycoides*, *Mycobacterium avium*, and *Mycobacterium phlei*. On isolation and purification it proved to give the typical streptomycin spectrum.

The results of this study permit the following conclusions: (1) not all strains of *S. griseus* are capable of producing streptomycin; (2) streptomycin-producing strains of *S. griseus* form active and inactive variants; (3) the inactive variants comprise two types, one being free from aerial mycelium and the other producing a pink tinge in the vegetative growth, the aerial mycelium being typical of *S. griseus*; (4) a medium enriched with streptomycin can be utilized for the isolation of fresh strains of *S. griseus* from natural substrates; and (5) a streptomycin-enriched medium can also be utilized for purifying active cultures of *S. griseus* from inactive variants.

Although these investigations did not result in obtaining superior strains of streptomycin-producing cultures of *S. griseus*, they established that streptomycin production is associated only with a certain few strains of *S. griseus* and that these strains may continuously form inactive substrains, and that by the use of suitable procedures it may be possible in time to isolate new and more potent strains of streptomycin-producing organisms.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

INDIANA BRANCH

Elkhart, Indiana, May 10, 1946

Limits of Effectiveness of Streptomycin in Arthritis of Rats.

H. M. Powell, Lilly Research Laboratories, Indianapolis, Indiana.

We have reported recently in a separate communication that streptomycin is an excellent chemotherapeutic agent against arthritic infections caused by pleuropneumonialike organisms in the rat. The culture we have used has been reported upon previously (J. Lab. Clin. Med., 1944, **47**, 523; J. Bact., 1944, 47, 523), and Dienes has reviewed this whole group (J. Bact., 1945, **50**, 441). Our former results, which were practically 100 per cent "cures," included use of 1,000 or 3,000 units of streptomycin per dose for 9 to 12 doses covering 3 or 4 days, and our test animals were white rats of about 100 g weight.

Amplified tests using less streptomycin, etc., now show that (a) 10 units streptomycin t.i.d. for 4 days is not effective; (b) 100 units t.i.d. for 4 days is a border-line therapy, curing only about three-fourths of the rats; (c) again, as previously noted, either 1,000 or 3,000 units t.i.d. for 3 or 4 days are effective; and (d) 3,000 units, but not 1,000 units, t.i.d., for 3 days with therapy starting one day after infection is effective. All chemotherapy heretofore reported, and herewith reported under (a), (b), and (c), started within an hour after intravenous infection, whereas therapy (d) began one day after infection.

A further experiment has been done in which the test culture has been injected into the foot pads, and 3,000 units streptomycin t.i.d. for 4 days has proved an effective therapy. Streptomycin appears to be a better chemotherapeutic agent than myochrysin against our pleuropneumonialike organisms.

Bacteriological Aspects in Minimizing Tranfusion Reactions.

Iva Dietz, Elkhart County Blood Bank, Elkhart, Indiana.

The important factors often overlooked or not stressed in blood transfusions are given careful consideration. Techniques for scrupulous cleaning of reusable apparatus are demonstrated; the importance of pyrogen-free water and solutions, and sterilization methods are explained.

Toxicity of Streptothricin.

Alfred R. Stanley, Research Department, Commercial Solvents Corporation, Terre Haute, Indiana.

Streptothricin produced in the pilot plant, and recovered in the laboratory as the sulfate and hydrochloride, was tested for toxicity to rabbits. Intravenous injection of either 5,000 or 20,000 units per kilogram of body weight twice a day resulted in a destruction of the mucous lining of the stomach, mottling of the liver and kidneys, and death of the animals. The same effects were produced by either salt. Feeding orally in capsules produced the same results. When two 60,000 to 85,000-unit capsules were given per day, the rabbits stopped eating on the second or third day, the nose was filled with mucus, and the chin was wet from drooling, indications of gastrointestinal disturbance.

Topical application of 30,000 units per day on scarified skin gave the same results as feeding, whereas the same application on unbroken skin had no effect. Intradermal injections of 6,000, 4,000, and 2,000 units produced hemorrhagic areas which increased in size for 6 days.

Testing of Germ-free Animals for Contamination.

James A. Reyniers, Laboratories of Bacteriology, University of Notre Dame, Indiana.

The term "germ-free" as applied to animals must mean freedom from demonstrable microbic contaminants within the limitations of the techniques which can be applied to determining their presence. Since the techniques are usually adequate for demonstrating the presence of recognized microorganisms, the problem really resolves itself into a theoretical question concerning the possible presence of those forms which cannot be demonstrated by microscopic, cultural, or chemical techniques. The presence of such microorganisms constitutes a special problem for future investigation.

The paper discusses this theoretical problem and offers examples from the work done in this laboratory on rats, guinea pigs, and chickens. It also includes the routine used for determining the presence of contaminations either in the living form or at necropsy. The final answer to the problem rests in the future and on a basis of being able to breed animals germ-free through a number of generations so that a pure strain can be developed.

PRODUCTION OF STREPTOTHRICIN IN SHAKE FLASKS AND 80-GALLON TANKS. *R. E. Bennett*, Research Department, Commercial Solvents Corporation, Terre Haute, Indiana.

REVIEW OF LITERATURE ON THE CARDIOLIPIN ANTIGEN IN THE SERODIAGNOSIS OF SYPHILIS. *Margaret Higgenbotham*, South Bend Medical Laboratories, South Bend, Indiana.

TEXAS BRANCH

AUSTIN, TEXAS, MAY 10 AND 11, 1946

EFFECT OF PARA-AMINOBENZOIC ACID ON THE INTESTINAL FLORA OF GUINEA PIGS. *Dorothy M. Whitney and Ludwik Anigstein*, Department of Preventive Medicine and Public Health, University of Texas School of Medicine.

Recent investigations on *p*-aminobenzoic acid (PABA) gave evidence of its wide range of action as an essential metabolite for bacterial growth, as a detoxicant, as a possible catalyst, and as a rickettsiostatic agent.

Since parenteral administration of PABA on experimental spotted fever was found ineffective, it was felt that a study of its effect on the intestinal flora by oral administration might throw light on the mechanism of its action.

The intestinal flora of normal guinea pigs was studied; this was followed by examination of the same animals after PABA was given. The fecal material was examined in direct smears, and aerobic cultures were made on various solid and liquid media.

Untreated guinea pigs showed a predominance of gram-positive bacilli and cocci in both direct smears and cultures. A total of 70 cultures, 54 of which were gram-positive, were isolated from a dozen apparently healthy guinea pigs. Of the 16 gram-negative strains, 11 were of the coliform type. There was a marked decrease of all bacteria after treatment with massive doses of PABA. After an interval of 4 PABA-free days the growth was still markedly restricted. No gram-negative, lactose-fermenting bacilli were found.

A PRACTICAL KEY FOR RAPID IDENTIFICATION OF THE COMMON SPECIES OF AEROBIC SPOREFORMING BACILLI. *Kenneth L. Burdon*, Department of Bacteriology and Immunology, Baylor University College of Medicine, Houston, Texas.

Previously suggested keys to the genus *Bacillus* based on a single morphological characteristic (spore size) or upon variable cultural features are inaccurate and impractical. The simple scheme presented here utilizes for differentiation a variety of properties found to be constant and readily demonstrable. It lists first the three species that ferment glucose, maltose, and mannitol (in bromcresol purple tryptose agar butt-slants). Among these, *Bacillus subtilis*, Ford, is identified by the fact that it grows well within 24 hours when an inoculated slant is incubated in a 56 C water bath. The two remaining species (*Bacillus megatherium* and *Bacillus circulans*) are distinguished by their general morphology and appearance when the fat is stained, as well as by their characteristic growth on potato slants. *Bacillus cereus* and *Bacillus mycoides*, which ferment glucose and maltose but not mannitol, are separated by the distinctive character of the mycelioid giant colony formed by the latter species on gelatin agar plates. The two species that ferment glucose but not maltose (*Bacillus mesentericus* and *Bacillus subtilis*, Marburg) are differentiated by the inability of the former to hydrolyze starch. Finally, *Bacillis brevis* is recognized by its failure to attack glucose.

Some Unusual Salmonella Types Found in Texas.

MacDonald Fulton, University of Texas School of Medicine, Galveston.

A report was made of 7 isolations of *S. panama* from man, 2 of *S. rubislaw* from two dysenteric monkeys, 3 of *S. habana* from diarrhea in adults and children, and 1 of *S. bareilly* from a child with diarrhea. Previous isolations of these species elsewhere were reviewed. These are the first strains of *S. rubislaw* to be reported from the monkey, and the second occurrence of *S. habana* to be reported anywhere.

A Presumptive Medium Differentiating Paracolon from Salmonella Cultures.

Martha Chilton, University of Texas School of Medicine, Galveston.

Carbohydrates fermented by various paracolon strains but not by *Salmonella* types were combined to give a new multiple-sugar broth. Fermentation in this broth within 24 hours indicated that a culture was probably a paracolon bacillus, if the use of polyvalent *Shigella* antiserum and a test for urea hydrolysis has excluded *Shigella* and *Proteus*, respectively. The medium contained 0.5 per cent each of adonitol, esculin, salicin, and sucrose. Kovacs' test for indole, performed on all cultures negative at 24 hours, further increased the detection of paracolon strains by this medium. In conjunction with the fermentation of lactose in agar slants containing 10 per cent carbohydrate, over 80 per cent of 252 paracolon strains tested were identified in 1 day.

The Selective Action of Penicillin in the Isolation of Brucella abortus from Milk.

Helen A. Lacy, L. J. Rode, and V. T. Schuhardt, Brucellosis Research Project of the Clayton Foundation at The University of Texas, Austin.

Pooled milk samples from the four quadrants of the udder were collected in sterile test tubes by hand milking from 564 cows. The cream from these samples was plated in duplicate on the usual gentian violet tryptose agar (GVTA) and on this medium containing 1 Oxford unit of penicillin per cubic centimeter (PGVTA). *Brucella abortus* colonies were isolated a total of 87 times from the two media. In 58 instances *B. abortus* was isolated on the PGVTA and not on the duplicate GVTA. In only 1 instance was the reverse of this situation true. In 20 instances the PGVTA plates showed more than 50 *B. abortus* colonies, whereas not one of the GVTA plates showed more than 50 colonies. In 12 instances the PGVTA plate showed more than 50 colonies of *B. abortus*, whereas not a single colony was found on the duplicate GVTA plate. *B. abortus* was isolated from 86 (15.24 per cent) of the 564 samples on PGVTA and from 29 (5.14 per cent) of the samples on GVTA.

Single Spirochete Infections in Experimental Relapsing Fever.

Martha Wilkerson and V. T. Schuhardt, The University of Texas, Austin.

Microcapillaries approximately ¼-inch long and 10 micra in diameter were filled with diluted blood serum containing spirochetes. These were mounted in saline and examined with the 4-mm objective using dark-field illumination. Those capillaries containing one spirochete were placed in the lumen of a hypodermic needle which had been partially plugged with agar and which was attached to a syringe containing saline. The contents of the syringe and needle were then injected into the peritoneal cavity of a rat.

Four of eleven rats so inoculated developed relapsing type infections. The infection sequences were followed by the examination of uniform (0.01 ml of a 1:20 dil.), daily, dark-field preparations of tail blood. The incubation period ranged from 5 to 7 days, and the number of relapses varied from 1 to 3. In general the infection sequences were similar to those resulting from tick bite infections.

Serological Aspects of the Relapse Phenomenon in Rats Infected with

SINGLE SPIROCHETES (BORELIA RECURRENTIS VAR. TURICATAE). *V. T. Schuhardt and Martha Wilkerson*, The University of Texas, Austin.

Antisera, collected from each of four rats 30 days after infection with single spirochetes, showed spirocheticidal activity against all antigenic varieties tested (attack passage, various relapse passage, and brain passage spirochetes of single-cell-infected and tick-infected rats). Thus, it would appear that each spirochete accomplishes all the possible antigenic variations characteristic of that particular strain during the course of the infection in the white rat.

Antisera collected from infected rats on the second day after the termination of each attack and relapse and on the thirtieth day after infection showed a constantly rising spirocheticidal titer against various strains of the spirochetes. This tends to confirm the concept that once spirochetes have taken part in an attack or relapse that particular antigenic variety cannot recur in subsequent relapses.

ANTIRICKETTSIAL ACTIVITY OF PARA-AMINOBENZOIC ACID (PABA): EVALUATION AND SIGNIFICANCE OF PABA BLOOD LEVELS IN GUINEA PIGS WITH REFERENCE TO SPOTTED FEVER TREATMENT. *Ludwik Aniystein and Dorothy M. Whitney*, Department of Preventive Medicine and Public Health, University of Texas School of Medicine.

An attempt was made in this study to investigate the correlation between the mode of administration of *p*-aminobenzoic acid (PABA) and its plasma concentration in the infected host with the course of spotted fever.

Significant differences have been found in the mode of administration since the compulsory oral or parenteral supply of the drug, resulting in free PABA levels as high as 43 to 69 mg per cent, had little effect on the progress oı the disease. Natural feeding, providing a frequent intake or an almost constant supply of PABA in small doses, corroborated previous results. Under these conditions, 0.3 g of PABA per 100 g body weight per day was found effective, particularly when protein (soya) and calcium were added.

In the plasma of normal and spotted fever guinea pigs not treated with any drugs, a substance was revealed which gave a color reaction matching PABA. This substance (DS) appeared only in traces of fasting guinea pigs; its level rose after normal feeding. No conjugated values were found.

PROTEINASE PRODUCTION BY BACILLUS SUBTILIS. *J. R. Stockton and Orville Wyss*, The University of Texas, Austin.

SOME EFFECTS OF STERILIZING GLUCOSE IN CULTURE MEDIA FOR THE GONOCOCCUS, WITH SPECIAL REFERENCE TO CYSTEINE. *C. E. Lankford*, The University of Texas Medical Branch, Galveston.

OBSERVATIONS ON DRUG SENSITIVITY OF COLIFORM ORGANISMS DURING ADMINISTRATION OF PHTHALYLSULFATHIAZOLE OR SUCCINYLSULFATHIAZOLE WITH PENICILLIN. *R. I. Wise, E. J. Poth, and Mary P. Slattery*, The University of Texas Medical Branch, Galveston.

FIELD AND LABORATORY INVESTIGATIONS OF EPIDEMIC INFLUENZA DURING THE WINTER OF 1945–46. *J. V. Irons and Oleta Beck*, State Department of Health, Austin.

THE USE OF ANTI-HUMAN-GLOBULIN SERUM AS A DEVELOPING TEST FOR INAPPARENT ANTIBODIES. *Sol Haberman, J. M. Hill, and Katharyn Willis*, Baylor University Hospital and Southwestern Medical College, Dallas.

AN AEROBACTER SP. PRODUCING A YELLOW PIGMENT. *Robert I. Wise, Mary P. Slattery, and E. J. Poth*, The University of Texas Medical Branch, Galveston.

BACTERICIDAL ACTION OF BROMINE. *Orville Wyss and J. R. Stockton*, The University of Texas, Austin.

INFLUENCE OF ETHER ANESTHESIA ON THE COURSE OF FIVE EXPERIMENTAL NEUROTROPIC VIRUS DISEASES. *Christine Zarafonetis, S. Edward Sulkin, and Cleo Housman Terry*, Southwestern Medical School, Dallas.

Prophylactic, Therapeutic, and Curative Tests in Avian Malaria with N1-(5-chloro-2-primidyl) metanilamide. *Wendell Gingrich and Eugenia Worley Schoch.* University of Texas Medical Branch, Galveston.

Survey of Typhus Rickettsiae in Rat Ectoparasites. *Lill R. Skinner, Ruth Keaton, and J. N. Murphy,* Texas State Department of Health, Austin.

Experimental Studies of Immunity to Pertussis in Infants. *Wallace Sako,* University of Texas Medical Branch, Galveston.

CENTRAL NEW YORK BRANCH

Ithaca, New York, June 20, 1946

Triple-Sugar Iron Agar (Hajna) and Lactose-Sucrose-Salicin Broth as Aids in the Identification of Salmonella. *Erwin Neter,* Children's Hospital and University of Buffalo.

Since certain *Proteus* and paracolon bacilli in Hajna's triple-sugar iron agar produce reactions similar to those of *Salmonella,* this culture medium was supplemented by a single broth containing 1 per cent lactose, 5 per cent sucrose, 1 per cent salicin, and phenol red as indicator (L.S.S. broth). In L.S.S. broth 37 *Salmonella* strains, representing 18 types, and 13 strains of *Proteus morganii* failed to produce acid. All 24 strains of *Proteus mirabilis,* recently isolated from feces of children with diarrhea, produced acid, 14 within 24 hours, 20 within 48, 22 within 72, and 2 after 72 hours. Eight strains, producing the *Salmonella* type of reaction in T.S.I. agar, formed acid in L.S.S. broth. Of 21 strains of paracolon bacilli, 17 caused acid formation in L.S.S. broth, namely, within 24 hours (10 strains), 48 hours (13 strains), and 72 hours (17 strains), respectively; 4 strains did not acidify the medium within 3 weeks. L.S.S. broth as an aid in the rapid and economic identification of *Salmonella* is discussed.

Studies of a Glycolytic Stimulant with Streptococcus faecalis. *C. E. Foust and I. C. Gunsalus,* Laboratory of Bacteriology, Cornell University.

During the course of differential fermentation studies, the rate of fermentation of cell suspensions and dried cells of lactic acid bacteria was found to decrease rapidly when the cells were stored. Yeast extract and a number of other natural materials were found to stimulate glycolysis of the cells.

Yeast extract and acid-hydrolyzed yeast extract have been fractionated and the nature of the active material has been partially determined. A number of the as-yet-unidentified growth factors described in the literature possess properties in common with the glycolytic stimulant observed in these studies.

Cells harvested from a phosphate-buffered glucose tryptone yeast-extract medium after 18 hours, final pH 4.5, showed a Q_G of about 100; these were stimulated by the addition of yeast extract to a Q_G of 200 to 400. Fructose is also fermented by these cells, though more slowly, and shows approximately the same degree of stimulation. Fractionation of acid-hydrolyzed yeast extract by butyl alcohol extraction left most of the activity in the aqueous residue. When further separation suggested that the histidine fraction possessed activity, this amino acid was tested and found to account for a portion of the yeast extract stimulation. In the presence of histidine the addition of glutamic acid or glutamine would further increase the stimulation.

Studies have been undertaken to determine whether this factor is acting directly on the glycolytic system and to locate the site of action.

The Production of Gaseous Nitrogen from Nitrate by the Legume Bacteria. *J. K. Wilson,* Department of Agronomy, Cornell University, Ithaca.

It is known that the legume bacteria under certain conditions can reduce nitrate to nitrite. If this occurs and certain organic and inorganic compounds are present in an acidic environment, the nitrous acid produced will react with such compounds and gaseous nitrogen will be liberated. Slopes containing nitrate that were inoculated with the bacteria and cultured for a few days and then plugged with some of the same medium developed sufficient gas to split the agar in about 36 hours. The gas was neither oxygen nor carbon dioxide, and was not inflammable. Since the legume bacteria reduce nitrate to nitrite, and since amines were supplied in the medium, it seems reasonable to conclude that the gas was nitrogen.

THE DARKENING OF MAPLE SYRUP DUE TO BACTERIAL ACTION. *C. S. Pederson and F. W. Hayward*, N. Y. State Agricultural Experiment Station, Geneva.

The chief factor in grading the quality of maple syrup is color; light-colored syrup usually has a more delicate maple flavor than dark-colored syrup.

The color of maple syrup depends upon the alkalinity of the sap and the invert sugar content. The growth of bacteria causes a temporarily increased alkalinity and the inversion of sucrose, which, in turn, result in darker-colored syrup. The bacteria grow at temperatures slightly above the freezing point and cause significant deterioration in color.

Cleanliness of equipment and rapid handling of the sap, therefore, are very important factors in the production of high quality, light-colored maple syrup.

THE DISAPPEARANCE OF HEMOPHILUS PERTUSSIS FROM INFANTS TREATED WITH STREPTOMYCIN. *Elizabeth Day and William L. Bradford*, University of Rochester, Rochester.

THE ANTIBACTERIAL ACTION OF STREPTOMYCIN IN EXPERIMENTAL BRUCELLOSIS OF GUINEA PIGS. *H. L. Gilman and W. R. LeGrow*, Veterinary College, Cornell University, Ithaca.

THE RESISTANCE OF THE GONOCOCCUS TO PENICILLIN IN VITRO. *Charles M. Carpenter, Leif G. Suhrland, and Martha Morrison*, University of Rochester, Rochester.

SELECTION OF SALMONELLA AND SHIGELLA CULTURES FOR TYPING. *W. H. Ewing*, Veterinary College, Cornell University, Ithaca.

THE RELATION BETWEEN ENZYME ACTIVITY AND VIABILITY IN DISINFECTION. *Martha Roberts and Otto Rahn*, Laboratory of Bacteriology, Cornell University, Ithaca.

THE OXALATE SALT OF P-AMINODIMETHYLANILINE, A REAGENT FOR THE OXIDASE TEST. *Charles M. Carpenter and Leif G. Suhrland*, University of Rochester, Rochester.

THE NATURE OF THE KERATIN FORMED IN RABBIT PAPILLOMATOSIS (SHOPE). *Henry W. Scherp and Jerome T. Syverton*, University of Rochester, Rochester.

SOME RELATIONS BETWEEN WEED KILLERS, INSECTICIDES, AND THE MICROORGANISMS IN THE SOIL. *J. K. Wilson and R. S. Choudri*, Department of Agronomy, Cornell University, Ithaca.

VIRUSES AND CANCER. (1 hour.) *Jerome T. Syverton*, University of Rochester, Rochester.

THE SEROLOGICAL RELATIONSHIP BETWEEN ERWINIA TRACHEIPHILA AND SPECIES OF SHIGELLA

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The immunological relationship of species and strains of species of the genus *Shigella* has been studied extensively (Weil, Black, and Farsetta, 1944; Wheeler, 1944; Weil, Barnes, and Binder, 1945; Wheeler and Stuart, 1946). Minor antigenic factors common to *Shigella* and certain *Salmonella* (Bornstein, Saphra, and Daniels, 1941), paracolon (Ferguson and Wheeler, 1946; Wheeler, Stuart, and Ewing, 1946), and coliform organisms (Stuart, Rustigian, Zimmerman, and Corrigan, 1943) also have been recognized. Slesinger and Elrod (1946) have also encountered cultures of *Proteus rettgeri*, from apparently typical Kligler slopes, which when spot-agglutinated in polyvalent *Shigella paradysenteriae* antiserum gave a definite reaction. For the most part all these organisms have a common habitat—the intestinal tract. They are occasionally found elsewhere but, except in the case of some coliforms, are never associated with plants.

The genus *Erwinia*, composed of the peritrichous, gram-negative plant pathogens, has been included in the family *Enterobacteriaceae*. The obvious similarity of the soft-rot members of this genus (*E. carotovora*, *E. aroideae*, etc.) to the *Escherichia-Aerobacter* group has long been recognized. There are, however, few studies to interrelate other *Erwinia* species with more remote members of the family. In a preliminary examination Elrod (1942) found many serological linkages between the genus *Erwinia* and the genera *Proteus*, *Salmonella*, *Escherichia*, and *Aerobacter*. Six soft-rot, four *E. amylovora*, one *E. lathyri*, and one *E. salicis* immune serums were employed. These were tested against 45 *Proteus*, 10 *Salmonella*, and 30 coliform cultures. With the *Proteus* there were 93 cross reactions (at 1:40 dilution or better) out of a possible 540 (17.4 per cent). The coliform cultures were less active as only 18 of 240 (7.5 per cent) reactions were evident. Of 110 possible reactions with *Salmonella*, 22 (20 per cent) were positive. The average cross titers were low. The coliform group averaged 530, *Proteus* 210, and *Salmonella* 125.

In a recent investigation (Elrod, 1946) pertaining to the inability of certain species of *Erwinia* to reduce trimethylamine oxide, a characteristic of many strains of *Erwinia* which is shared in the *Enterobacteriaceae* only by some members of the genus *Shigella*, it was noted that among these was *Erwinia tracheiphila*. A spot agglutination test of a suspension of this organism with polyvalent Flexner serum gave a definite reaction. This did not occur with other trimethylamine-negative *Erwinia* species, i.e., *E. amylovora*, *E. phytophthora*, *E. solanisapra*, and some strains of *E. carotovora* and *E. aroideae*. It seemed expedient to investigate further this agglutination reaction to determine, if possible, whether or not primary or secondary *Shigella* factors were involved and to ascertain the frequency of common factors between species of *Shigella* and *E. tracheiphila*.

EXPERIMENTAL

Three strains of *Erwinia tracheiphila* were available for this study.[1] They have been indicated as T1, T3, and T4. Needle puncture inoculations revealed that all three were pathogenic on the common cucumber. The plants showed evidence of wilt in 6 to 7 days and all infected runners were dead in 12 to 14 days.

From previous platings colonies were selected that were smooth, easily homogenized in 0.9 per cent saline, and not agglutinated by trypaflavine (1:500). These were used for the preparation of the antiserums. Hyperimmune serums were made for 2 of the 3 isolates (T1 and T3). The antiserums were prepared in two ways. One pair of rabbits was injected with living saline suspensions of the respective organisms. Another two were immunized with suspensions that had been boiled for 60 minutes to destroy the flagellar antigens.

The agglutination tests were set up in the usual way, incubated for 18 hours at 56 C or 45 C, followed by several hours' incubation in the cold. At all times the agglutination occurred in the granular fashion usually attributed to somatic factors. Reciprocal absorption showed the two strains of *Erwinia tracheiphila* (T1 and T3) used for immunizing to be serologically identical. Elrod (1942) found that four strains of the cucumber pathogen were antigenically alike.

Cross-agglutination experiments were conducted using *Erwinia tracheiphila* suspensions as antigens and unabsorbed *Shigella* serums.[2] These results are tabulated in table 1. It was found that agglutinating suspensions of *E. tracheiphila* that had been boiled for 60 minutes gave sharper results and therefore were used exclusively. Although the degree of agglutination of the plant pathogens never equaled that of the homologous *Shigella* strain, it was often considerable and marked. This was true in *S. paradysenteriae* types I, III, IV, P288, and P143 antiserums and to a lesser extent in serums prepared against type VI, 170, *S. dysenteriae*, and *S. alkalescens*. The degree of agglutination of all three isolates was about the same. Of the available *Shigella* antiserums only *S. paradysenteriae* type II(VII), V, and D19, and *S. ambigua* failed to agglutinate the *E. tracheiphila* cultures. All the *Shigella* antiserums employed had homologous titers of 1,280 or 2,560.

The cross reactions of the various *Shigella* species in *Erwinia tracheiphila* antiserums are listed in table 2. The two antiserums employed had homologous titers of 1,280 to boiled suspensions. Those rabbits immunized with living motile cultures produced serums that agglutinated the homologous living suspensions of T1 and T3 to 10, 280 and 20, 560, respectively. Most of the agglutinations were flagellar, and the resulting *Shigella* crosses were not so strong as in the serums prepared with the heated suspensions. For the most part the reactions in the anti-*tracheiphila* serums reciprocated those that occurred in the *Shigella* antiserums. Flexner type IV gave the strongest reaction (640), whereas type I, III, X, Y, and P288 were evident but weaker. *Shigella dysenteriae*, *S. alkalescens*, and Sachs' type Q1167 gave definite reactions. *Shigella paradysenteriae* types II(VII), V, VI, 170, P143, P274, D1, and D19 failed to agglutinate. This was true of other types of *Shigella*—*S. sonnei*, *S. ambigua*, *S. etousae* (Lavington type), and Sachs' Q454.

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[1] I am indebted to Dr. S. P. Doolittle, Bureau of Plant Industry, Beltsville, Maryland, for these cultures.

[2] These antiserums were prepared by Miss Marion Orcutt, The Rockefeller Institute for Medical Research, Princeton, New Jersey.

Absorption experiments were conducted on one of the *Erwinia tracheiphila* antiserums. The absorption by any of the reactive *Shigella* organisms removed the antibodies for the absorbing organism in each case, but the homologous titer never was affected (table 3). By and large, these absorptions had only minor effects on the remaining *Shigella* reactions. Likewise absorption of P288, IV, and *S. dysenteriae* antiserums by *E. tracheiphila* (not tabulated) did not appear to affect the homologous reactions. It is to be assumed then that we were dealing with a multitude of secondary antigens.

TABLE 1

Cross-agglutination reactions of Erwinia tracheiphila in Shigella antiserums

ANTISERUM	HOMOLOGOUS TITER	CULTURE OF E. TRACHEIPHILA AGGLUTINATED		
		T1	T3	T4
S. paradysenteriae				
I	1,280*	320	160	320
II(VII)	1,280	<40	<40	<40
III	2,560	320	160	320
IV	1,280	320	320	160
V	2,560	<40	<40	<40
VI	2,560	160	160	160
P288	2,560	640	320	320
170	1,280	80	80	80
P143	1,280	320	160	160
D19	2,560	<40	<40	<40
S. dysenteriae	2,560	160	160	160
S. ambigua	1,280	<40	<40	<40
S. alkalescens	1,280	160	160	160

* Represents reciprocal of highest dilution showing macroscopic agglutination.

Nevertheless, in an effort to determine whether the common components could be identified as known *Shigella* antigens (and not having the necessary absorbed serums at hand), we sent heated suspensions and cultures of the cucumber pathogen to Dr. K. M. Wheeler. He reported in part as follows: "Suspensions T1 and T3 and culture T1 [the only one submitted] gave just about the same reactions.... I obtained a positive agglutination with the polyvalent Flexner serum, *S. alkalescens* and polyvalent Boyd's group serums. None of the agglutinations were strong, however. Single factor tests were positive with

Flexner group 3 serum and Boyd P288 serum. . . . I doubt if the antigen could be identified with any of the known components of the dysentery group."

TABLE 2

Cross-agglutination reactions of Shigellae in Erwinia tracheiphila antiserums

ORGANISM AGGLUTINATED	ANTISERUM T1	ANTISERUM T3
S. paradysenteriae		
I	80	160
II(VII)	<40	<40
III	160	80
IV	640	320
V	<40	<40
VI	<40	<40
X(VII)	40	40
Y(VIII)	160	160
P288	80	160
170, P143, P274, D1, D19, *S. sonnei*, *S. ambigua*, *S. etousae*	<40	<40
S. alkalescens	160	160
Sachs' Q454	<40	<40
Sachs' Q1167	80	80
E. tracheiphila (T1)	1,280	1,280
E. tracheiphila (T3)	640	1,280

TABLE 3

Absorption of Erwinia tracheiphila (T1) antiserum by Shigella

ORGANISM AGGLUTINATED	BEFORE ABSORPTION	ABSORBED WITH:							
		I	III	IV	Y	P288	*S. dysenteriae*	*S. alkalescens*	Q1167
S. paradysenteriae									
I	80	<40	40	<40	<40	80	80	40	80
III	160	80	<40	<40	40	80	160	80	80
IV	640	160	320	<40	80	160	320	320	320
Y	160	80	40	<40	<40	80	160	160	80
P288	80	80	80	80	80	<40	40	40	80
S. dysenteriae	160	160	160	160	160	160	<40	160	80
S. alkalescens	160	160	160	160	160	80	80	<40	80
Sachs' Q1167	80	80	80	80	80	80	<40	<40	<40
E. tracheiphila (T1)	1280	1280	1280	1280	1280	1280	1280	1280	1280
E. tracheiphila (T3)	640	640	640	640	640	640	640	640	640

Cultures and suspensions were submitted, also, to Major P. R. Carlquist of the Army Medical school. He, too, found that all three of the strains reacted in *Shigella alkalescens* type I serum and that T1 reacted in Boyd P288 serum. He employed a larger group of serums and found that all the suspensions gave a ++ or +++ reaction with *S. dysenteriae* serum, and that T3 and T4 reacted weakly in Sachs' Q1167 antiserum.

DISCUSSION

It is evident that the reactions herein recorded between *Erwinia tracheiphila* and *Shigella* species, although numerous, are minor. Wheeler (1944) in his studies of *Shigella paradysenteriae* has designated many group components among types of this species. Weil *et al.* (1944), on the other hand, refer to these components only as secondary antigens. Our absorption studies and the tests made in other laboratories with specific absorbed serums have indicated that the factors were not identifiable with the primary *Shigella* factors. It is apparent that the interacting components comprise only small portions of the antigenic pattern of *Shigella* types and *E. tracheiphila,* and because of our incomplete knowledge of such secondary factors cannot be identified positively with known *Shigella* components. Dr. Wheeler found some evidence that his group 3 component was responsible for a part of the reaction. This portion is not evident in P288, *S. alkalescens,* and other reacting organisms and is only one of several small factors.

Erwinia tracheiphila is a well-defined plant pathogen. It satisfies all the requirements of the genus *Erwinia* as recently defined by Borman, Stuart, and Wheeler (1944). Like *Shigella* and *Proshigella* it is anaerogenic in its action on glucose and other carbohydrates, but unlike the members of these genera *E. tracheiphila* readily attacks salicin. Although it was omitted by the foregoing authors in their consideration of the *Erwinia,* it definitely deserves a place with *E. amylovora* and *E. salicis.* The bacterium is pathogenic to many, but not all, of the *Cucurbitae,* and in nature is transmitted by the cucumber beetle. It easily survives this insect environment, passing through the alimentary tract without harm. It has been shown to overwinter in the adult beetle, and there is evidence of multiplication in the insect vector. Primary infections in the spring originate from the feeding punctures of such beetles (Rand and Cash, 1920; Rand and Enlows, 1920). The limited host range, its dependence upon insect transmission in the natural state, the relative biochemical inactivity, and the apparent serological homogeneity of *E. tracheiphila* indicate a high degree of specialization.

One may hypothesize, also, that *Shigella* represents an advanced state in the evolutionary progress of the *Enterobacteriaceae.* Here is found a normal habitat limited to the intestinal tract, a relative biochemical inactivity, especially in certain species, and an antigenic make-up less diverse than the more primitive forms. Although the evolutionary paths of *Shigella* and *Erwinia tracheiphila* have taken different directions one may postulate that there was a single origin which manifests itself now in common antigenic fragments. In view of the morphological and biochemical similarity of the two it is considered that the common antigenic factors do represent the remnants of a common origin. The phenomenon of para-agglutination, as discussed by Mackie (1939), which so often has been considered in heterologous reactions between intestinal organisms of different genera, is not a factor in this case. As comparative studies are made of groups within the *Enterobacteriaceae,* this common origin seems more and more evident. The immunological relations between *E. tracheiphila* and species of *Shigella* further emphasize the intergraded relationships that exist throughout the family.

SUMMARY

It was observed that suspensions of *Erwinia tracheiphila* agglutinated in many *Shigella* antiserums. Likewise a number of *Shigella* organisms reacted in *E. tracheiphila* antiserum. The resulting titers were not high but reached on occasion 50 per cent of the homologous reaction.

In specific *Shigella paradysenteriae* antiserums it was not possible to identify the active *Shigella* factors. Absorption of *E. tracheiphila* antiserum revealed that a multiplicity of minor antigens were involved.

The possibility of a common origin for *Erwinia tracheiphila* and the genus *Shigella* is discussed.

ACKNOWLEDGMENT

I wish to thank Dr. K. M. Wheeler, State Department of Health, Hartford, Connecticut, and Major P. R. Carlquist, Chief of the Bacteriology Section, Army Medical School, Washington, D. C., for their efforts in attempting to identify the specific factors.

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THE MOUNTING OF BACTERIA FOR ELECTRON MICROSCOPE EXAMINATION

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The use of the electron microscope in bacteriology has yielded much new information regarding the structure of the bacterial cell (Mudd, 1944; Mudd and Anderson, 1944). On the other hand, the possibility has always been recognized that certain relationships might be altered by artifacts introduced by the treat-

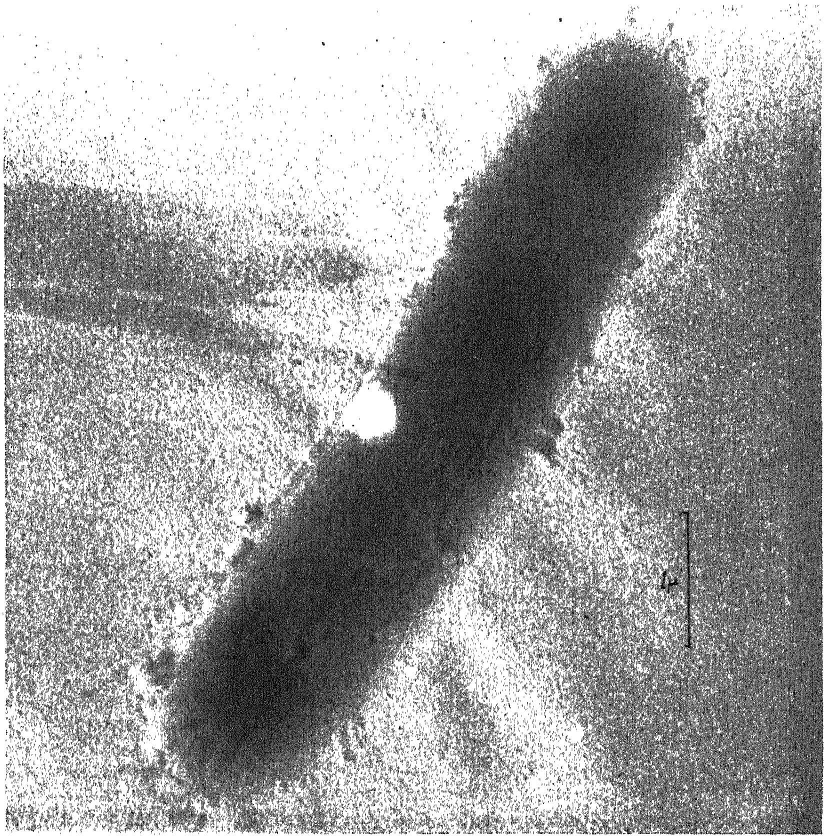

FIG. 1. AN ELECTRON MICROGRAPH OF ESCHERICHIA COLI MOUNTED ON COLLODION FROM A DISTILLED WATER SUSPENSION AND HEAVILY SHADOWED WITH GOLD TO SHOW THE WRINKLED SURFACE

ment which the organisms undergo in the preparation of the specimens for the electron microscope. Recent work in this laboratory has provided some information regarding the existence and nature of such artifacts and has led to a new method of preparation that avoids many of the difficulties in the case of some organisms that can be cultured on solid media.

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[1] The authors wish to thank Dr. Stuart Mudd of the University of Pennsylvania for his interest and for reading the manuscript of this note.

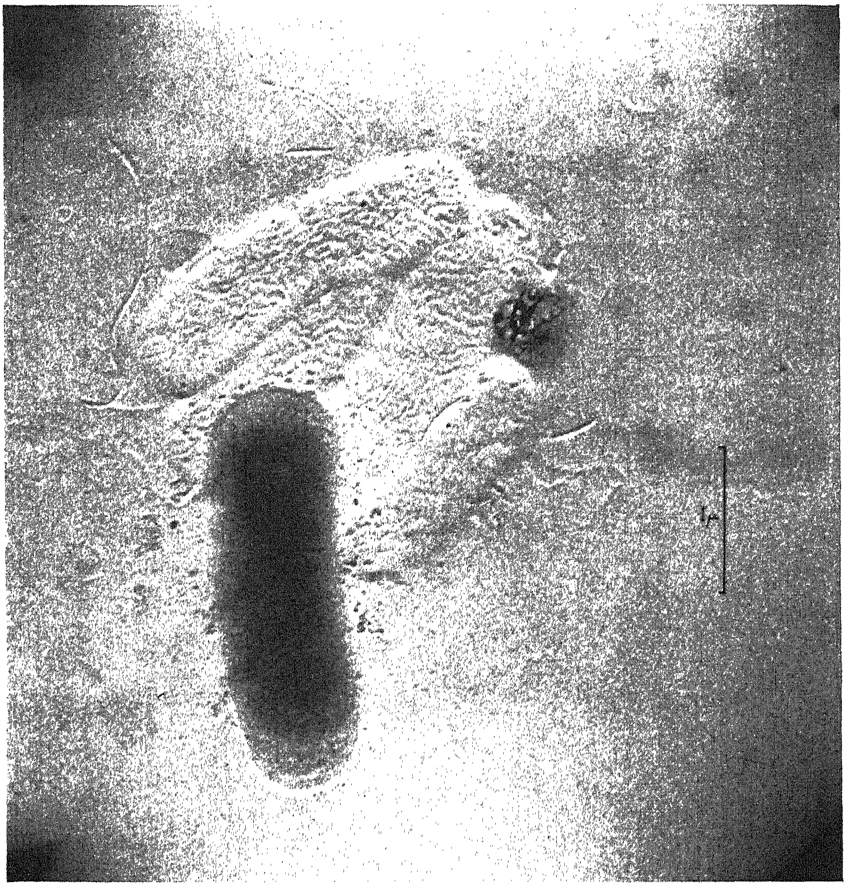

FIG. 2. AN ELECTRON MICROGRAPH OF A GOLD-SHADOWED REPLICA OF SERRATIA MARCESCENS

The bacteria were mounted on a glass slide from a distilled water suspension. The replica was made of this mount and subsequently shadowed. One cell in the field shown adhered to the collodion.

The ordinary method of mounting bacteria for examination in the electron microscope involves suspending the organisms in distilled water, placing a small drop of the suspension on a specimen screen provided with a supporting membrane, and allowing the water to evaporate. There are several points in this procedure at which artifacts may be produced, including the initial disturbance of the bacteria on removal from the culture, the effects of the distilled water, and the subsequent desiccation. Placing the mounted specimen in the vacuum of the electron microscope and subjecting it to intense electron bombardment may also introduce artifacts.

Relatively little work has been done on the determination of the exact nature and extent of these artifacts. From an investigation of the effects of distilled water on *Bacillus subtilis* and *Escherichia coli* it was concluded that, although there is a steady disintegration of the cells in suspension, the first few minutes do not appreciably affect the electron microscope images (Hillier and Kurkjian, 1944). This work did not exclude the possibility that there is a sudden change of morphology when the suspension is made. A light microscopic investigation of the effect of vacuum on bacterial films gave negative results within the resolu-

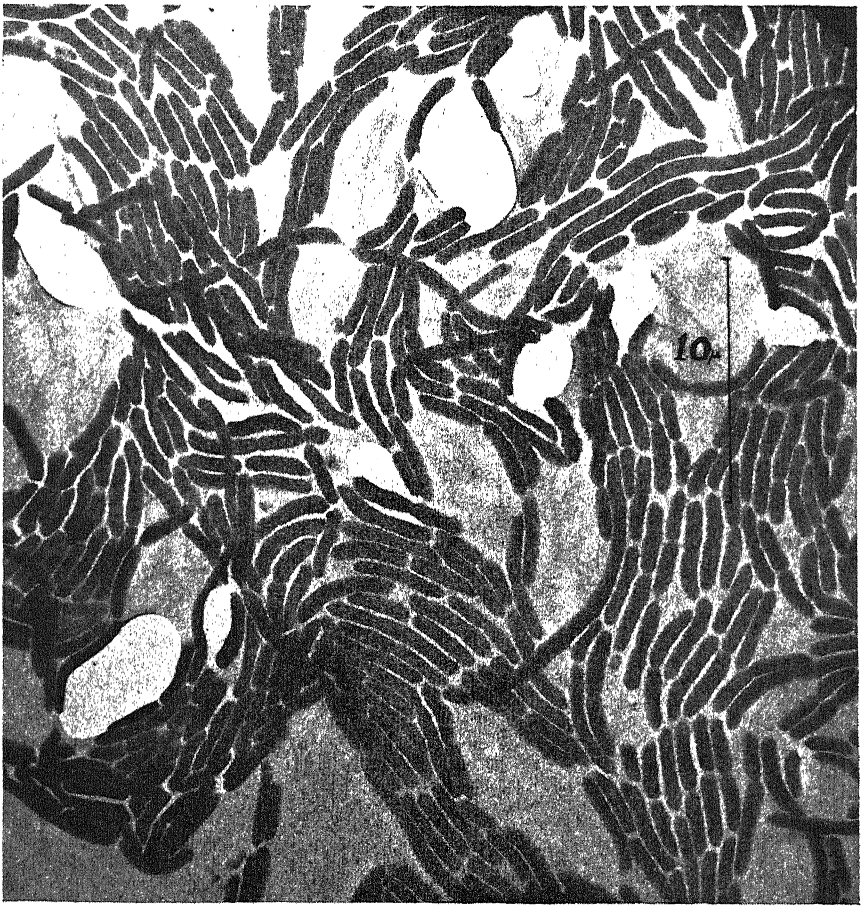

Fig. 3. A Low Magnification Electron Micrograph of Serratia marcescens Prepared According to the Method Described

This specimen was taken from a 5-hour agar plate culture tion limits of the instrument. High magnification observations in the electron microscope have indicated that, except at extremely high electron intensities, there is no change in morphology as bacteria are brought into the electron beam.

In the present work a further investigation of the artifacts in electron micrographs of bacteria has been conducted using various modifications and combinations of the replica technique of Schaefer and Harker (1942) and the shadowing technique of Williams and Wyckoff (1946). It was observed first that many types of bacteria presented a characteristic wrinkled and flattened appearance when mounted in the conventional way and subsequently heavily shadowed with gold (figure 1). It was then found that gold-shadowed replicas of bacterial films from distilled water presented the same appearance (figure 2). Now the cells of which replicas were obtained had *not* been in a vacuum, since, in this

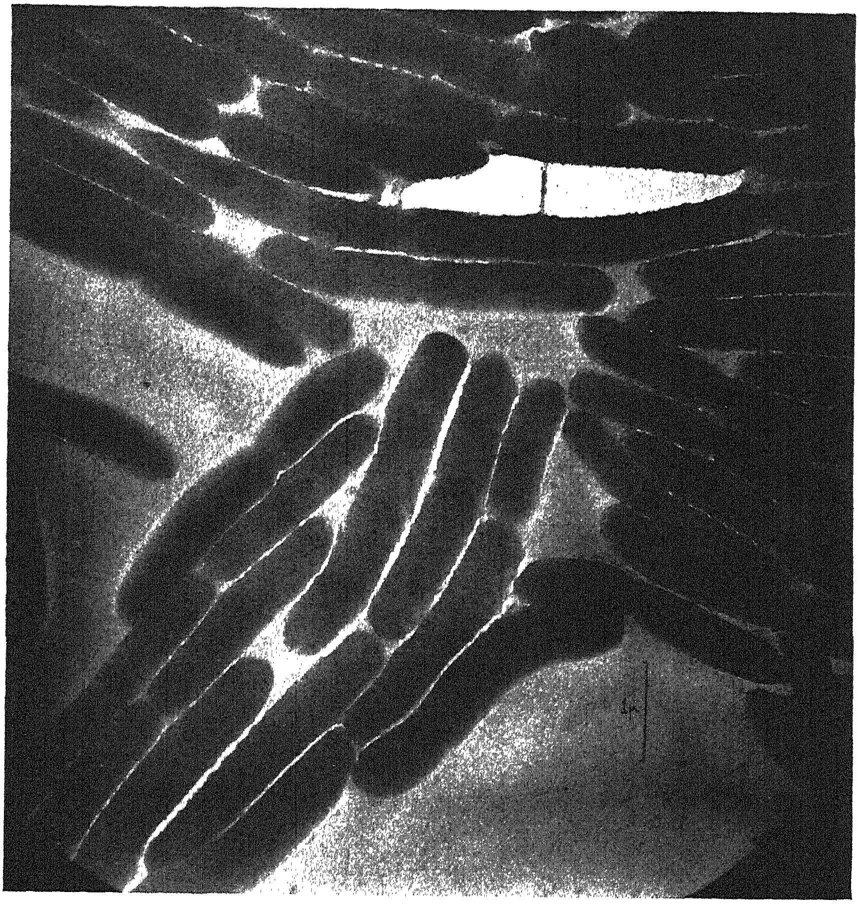

FIG. 4. HIGHER MAGNIFICATION ELECTRON MICROGRAPH OF A SIMILAR PREPARATION SHOWING SOME INTRACELLULAR GRANULES AND A SLIGHT EVIDENCE OF SHRINKAGE

technique, a collodion replica was made of a dried film on a microscope slide *before* shadowing. It can be concluded, therefore, that the wrinkling and flattening is a result of the initial desiccation and that introduction into the vacuum of the microscope introduced little, if any, further change. Replicas of dried bacteria which had been dispersed initially in a fixing agent showed the cells to be smooth and rounded.

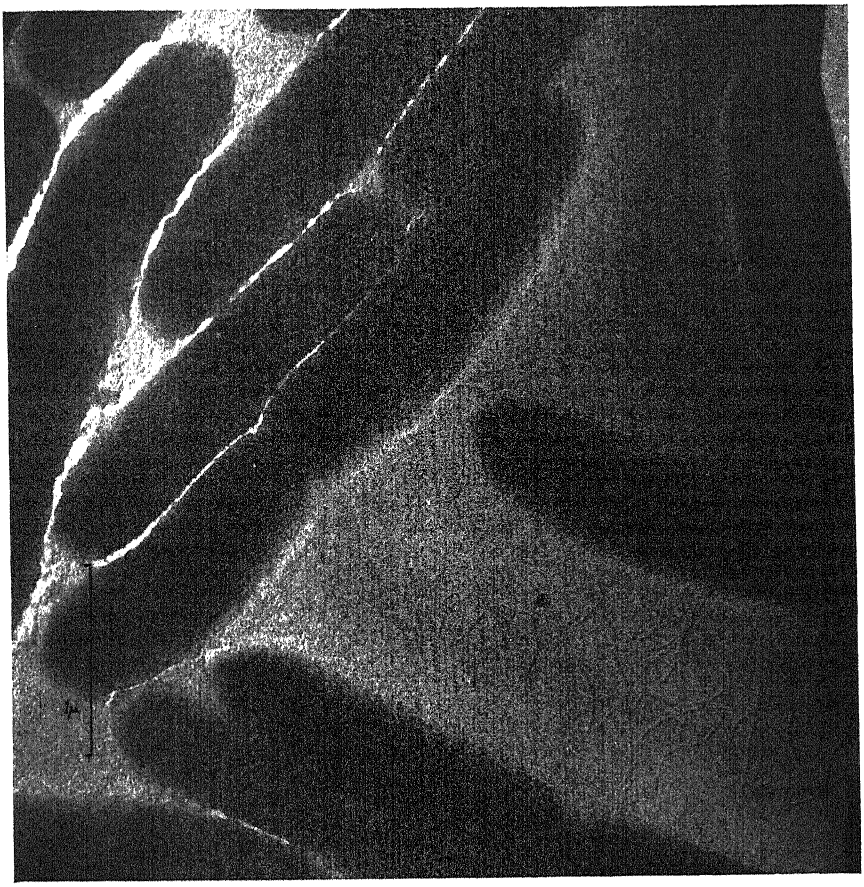

FIG. 5. AN ENLARGEMENT OF PART OF FIGURE 4 SHOWING THE GROWTH OF THE FLAGELLA ON THE SURFACE OF THE AGAR AND A FINELY DIVIDED MATERIAL SURROUNDING THE BACTERIAL CELLS

These observations led to an attempt to obtain a replica of a growing agar plate culture. The technique which was tried consisted simply of flowing a 1 per cent solution of collodion in amyl acetate over a small and young colony, allowing it to evaporate to dryness, floating the collodion film off on a water surface, and shadowing the replica surface with gold after mounting and drying. The technique produced the unexpected results shown in figures 3 to 5. Instead of obtaining a replica we found that a surface layer of the culture was removed *intact* by the collodion and that the subsequent handling produced little, if any, change in the appearance of the preparation.

It now appears that, for the study of some bacteria which can be grown on agar-like solid media, this technique has advantages over the conventional one. The most obvious advantage comes from the possibility of studying the bacteria in the environment in which they were grown. Flagella and bacterial excretions which are usually distorted or lost in the older method of preparation can now be studied without disturbing their exact relationship to the bacteria in the growing colony. In fact, the appearance of the flagella in figure 5 suggests to the authors that they may perform functions other than as organs of locomotion. Since the physical relationship of the cells in the growing colony is preserved, it should now be possible to make an accurate correlation between changes in morphology and the processes of cell division. Although the method does not inherently eliminate the possibility of artifacts due to contact between the cells and distilled water, none of the characteristic artifacts have been observed—a fact which may possibly be due to a fixing action of the collodion solution.

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SMALL COLONY VARIANTS OF ESCHERICHIA COLI

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Small colony variants of several species of bacteria have been described (Hadley, Delves, and Klimek, 1931; Hoffstadt and Youmans, 1932; Koser and Dienst, 1934; Duff, 1937; Haddow, 1938; Kuhn and Sternberg, 1931). Their recovery has usually been a matter of chance, because of their sporadic occurrence, with one exception: Youmans (1937, 1942) has reported the regular appearance of small colony variants in 17 strains of staphylococci in response to barium chloride. References to small colony variants of *Escherichia coli* are rare. Hadley, Delves, and Klimek (1931) mention briefly that "G" forms similar to "G" forms of the *Shigella* group extensively studied by them were recovered from 2 strains of *E. coli;* and Kuhn and Sternberg (1931) found small colony variants in 45 strains of *E. coli*, among other organisms. The present report deals with the production of small colony variants of *E. coli* with 2-methyl-1,4-napthoquinone and is believed worth recording because of the frequency of their appearance, the purity of the phase obtained, and their possible significance in regard to the mechanism of the antibacterial action of this quinone.

METHODS

MacLeod's (1940) synthetic medium dispensed in accurately measured amounts in 8-by-1-inch test tubes was used. 2-Methyl-1,4-naphthoquinone was dissolved and diluted aseptically in 80 per cent acetone and added in 1-ml amounts to 24 ml of medium either before or after the medium was autoclaved at 10 pounds' pressure for 20 minutes. Dilutions of the quinone were adjusted to give a series of final concentrations varying from one which was bactericidal in 24 hours to one which permitted normal growth in 24 hours. The final dilutions usually employed were 0.0025, 0.00125, 0.000625, and 0.0003125 per cent. Acetone and water controls were included in each series. To eliminate the possible effects of acetone, the water-soluble sodium bisulfite addition product of 2-methyl-1,4-naphthoquinone was employed in a similar manner. Although minimum effective bacteriostatic and bactericidal concentrations of this product were somewhat variable (Colwell and McCall, 1946), 0.04, 0.02, 0.01, and 0.005 per cent usually sufficed for the desired range.

Four strains of *E. coli* were employed. Three were old stock laboratory cultures,[1] and one was freshly isolated from human feces. All were purified by repeated plating and picking of single colonies, and before use purified strains were subcultured at least 6 times in asparagine medium to eliminate possible traces of peptone. Three strains were non-sucrose-fermenters; the fourth was *E. coli-communior*. The freshly isolated strain and one of the old stock cultures were in the smooth phase; the remaining two cultures gave rise to both smooth and intermediate RS colonies even after repeated picking of single S colonies. This fact possibly indicated a tendency toward instability and dissociation in the latter two strains. The inoculum was 0.1 ml of a 24-hour culture diluted 1:4 (approximately 800,000 to 1 million organisms per ml of culture medium). Bacteriostasis was observed daily by visual observations of turbidity, and each tube was streaked daily on nutrient agar without quinone for the study of colonial types. Sugar fermentations were studied in peptone water containing 1 per cent of glucose, lactose, sucrose, or mannitol, gas production being observed in small inverted tubes.

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[1] Two were obtained through the courtesy of Dr. A. W. Walker of the Department of Bacteriology, Northwestern University Medical School, Chicago, Illinois, and the third from Dr. Harriman of the Research Laboratories of Armour and Company, Chicago, Illinois.

RESULTS

One tube in the naphthoquinone series was usually clear in 24 hours and very faintly turbid in 48 to 72 hours, the turbidity increasing slightly on continued incubation. Subcultures from these tubes on nutrient agar without quinone usually resulted in 24 hours in a barely visible haze of translucent, pinpoint colonies, which increased slightly in size and visibility on longer incubation. Sometimes myriads of these small colony forms would be interspersed with an occasional colony of normal size. Transplants of single colonies of small colony variants to fresh agar without quinone at about 5-day intervals frequently resulted in maintaining this phase over long periods of time if the fourth or fifth transplant was stored in the refrigerator and subsequently transplanted at infrequent intervals.

Small colony variants were recovered in this manner from all four strains of bacteria. The percentage of successful trials was greater with the three old stock strains than with the freshly isolated strain. Water and acetone controls never produced the picture described; an occasional small colony in agar streaks of control tubes was the exception, and picking and restreaking such a colony (always larger and more opaque than the small colony variants produced with the quinone) invariably resulted in pure cultures of "normal" colonies. The water-soluble sodium bisulfite addition product of 2-methyl-1,4-naphthoquinone was as efficacious in producting small colony variants as the quinone itself. In general, greater success in the production of small colony variants attended the aseptic addition of the quinone to sterilized, rather than to unsterilized, medium; although the variants were obtained with medium autoclaved after addition of the quinone, and sufficiently often to prove that it was possible to produce them in this manner, strains so recovered were somewhat less stable.

Morphologically, small colony variants were usually indistinguishable from their parent strains. Occasionally a strain grew in long filaments, apparently lacking normal powers of division.

When nutrient broth was substituted for the chemically defined asparagine medium, small colony variants of *E. coli* were not obtained. A strain of *Staphylo-*

coccus aureus in 2-methyl-1,4-naphthoquinone nutrient peptone broth produced only occasional unstable small colony variants. Brief trials with strains of *Aerobacter aerogenes*, *Bacillus subtilis*, and *Bacillus mesentericus* in asparagine medium containing 2-methyl-1,4-napthoquinone failed to produce small colony variants.

Reversion of small colony variants of *E. coli*, stable on the original plate, began not infrequently on the first transfer to fresh agar. Usually one or two large colonies would appear in the midst of innumerable tiny colonies, and a pure culture of reverted forms was procured only by repeatedly picking the larger colonies to broth and restreaking. Rapid subculture and cultivation in liquid mediums, especially those containing an enrichment such as tryptose or glucose, favored reversion. Stock cultures of small colony variants transferred at infrequent intervals and kept refrigerated often remained stable over a period of months, but descendants of the same single colonies would revert on rapid subculture. Partial reversion was usually not difficult; complete reversion, that is, obtaining pure cultures of reverted forms, was sometimes laborious and time-consuming.

TABLE 1

Biochemical characteristics of small colony variants of Escherichia coli compared with those of original and reverted strains

STRAIN	GLUCOSE		LACTOSE		MANNITOL		SUCROSE		INDOLE	NO2	METHYL RED	VOGES-PROSK.	EACH TEST STREAKED ON AGAR
	A*	G	A*	G	A*	G	A*	G					
E. coli I	4.75	+	5.05	+	4.85	+	7.98	−	+	+	+	−	100% S
E. coli I S.C.V.	5.3	−	5.4	−	7.0†	−	7.2	−	−	−	+	−	100% S.C.V.
E. coli I Revert	4.75	+	5.05	+	4.85	+	7.75	−	+	+	+	−	100% S
E. coli II	4.5	+	4.7	+	4.6	+	5.2	+	+	+	+	−	RS, S
E. coli II S.C.V.	5.3	−	5.2	−	5.7	−	5.6	−	+	+	+	−	100% S.C.V.
E. coli II Revert	4.65	+	5.2	+	4.85	+	5.65	+	+	+	+	−	RS, S
E. coli III	4.6	+	5.05	+	4.9	+	7.5	−	+	+	+	−	RS, S
E. coli III S.C.V.	5.1	−	5.2	−	6.9†	−	6.9	−	−	−	+	−	100% S.C.V.
E. coli III Revert	4.8	+	5.2	+	5.1	+	7.3	−	+	+	+	−	RS, S
E. coli IV	4.35	+	4.6	+	4.5	+	7.5	−	+	+	+	−	100% S
E. coli IV S.C.V.	5.25	−	5.4	−	5.8	−	6.8	−	+	faint +	+	−	100% S.C.V.
E. coli IV Revert	4.4	+	4.5	+	4.55	+	7.4	−	+	+	+	−	100% S

* Figures stand for pH as measured by glass electrode.

† Growth doubtful.

The stability of some strains of small colony variants made possible a comparison of some of their biochemical characteristics with those of the parent and reverted strains. Beginning reversion was detected in these tests by streaking on an agar plate at the time of reading the test; only those cultures proved in this manner to consist completely of colonies in the proper phase were included in the results shown in table 1. Original cultures, reverted cultures, and small colony variants were all methyl-red-positive and Voges-Proskauer-negative. Original and reverted strains all reduced nitrates to nitrites and all were indole-positive. Small colony variants from two of the four coli strains reduced nitrates and produced indole; those from the remaining two strains were indole- and nitrite-negative.

Sugar fermentation by small colony variants was interesting in that all produced acid, but failed to produce gas, from the same sugars as the parent strains except for two which apparently failed to multiply in mannitol medium. Acid production as measured by the glass electrode, however, was less than that produced from the same sugars by parent and reverted strains. Original and reverted strains growing in plain or sucrose broth produced alkaline pH's; small colony variants did not change the pH of plain or sucrose broth significantly.

DISCUSSION

Interest in "G" forms of bacteria has subsided somewhat since the challenge by several later investigators of Hadley's early theory (1931) that they were stages in the life cycle of the organism. Confusion attending the small colony variant has taken some order with the evidence of Koser (1930, 1934), Swingle (1935), Youmans (1937, 1942), Chinn (1936), and others that these organisms are merely cells of lowered metabolic activity due to interference or destruction of enzymes by conditions somewhat unfavorable for growth. The following points lend credence to this view: (a) The conditions under which small colony variants are found, as in aged cultures (Hadley, Delves, and Klimek, 1931; Koser and Dienst, 1934; Swingle, 1935) and as in the presence of lithium chloride (Hadley, Delves, and Klimek, 1931; Koser and Dienst, 1934; Swingle, 1935), barium chloride (Youmans, 1937, 1942), bacteriophage (Kuhn and Sternberg, 1931), phenol (Duff, 1937), penicillin (Schnitzer, Camagni, and Buck, 1943), and 2-methyl-1,4-naphthoquinone, are somewhat inhibitory to bacterial growth. (b) At least two of the foregoing agents (barium chloride and 2-methyl-1,4-naphthoquinone) favor the development of the small colony variant in medium poor in growth factors (meat-extract-activator peptone medium and synthetic asparagine medium); the same agents in more nutritious broths fail to induce this type of variation. (c) In the present work with *E. coli,* the slow, meager growth of the small colony variant, the failure to evolve gas from fermented sugars, and lesser acid production from fermented sugars—all point to interference with normal enzyme function resulting in lowered metabolic activity. (d) It has been shown that the antibacterial activity of 2-methyl-1,4-naphthoquinone is due, at least in part, to interference with essential sulfhydryl systems or metabolites (Colwell and McCall, 1945, 1946). Sublethal concentrations of the quinone apparently result in sufficient enzyme interference to prevent normal growth, and the small colony variant results.

An explanation for all types of bacterial variation which readily presents itself is that the variant is present in the original culture in numbers so small as to be undetectable by ordinary methods, but because of its greater resistance to unfavorable conditions it survives and multiplies when normal cells cannot. This theory is not necessarily at variance with the evidence that small colony variants are forms of lowered metabolic activity. It is more difficult, however, to provide unequivocal evidence that their greater resistance is the reason for their detection. Small colony variants of *Staphylococcus aureus* have been obtained from cultures arising from a single cell (Hoffstadt and Youmans, 1938). In view of the knowledge that a single cell does not necessarily produce homogeneous progeny, however, it does not appear that small colony variants arising from single cell cultures provide the answer to the question of their resistance. Hadley, Delves, and Klimek (1931) state that their dysentery "G" forms were resistant to bacteriophage for the parent strain, but that they had the same thermal death point as normal strains. Schnitzer, Camagni, and Buck (1943) state that small colony variants of staphylococcus produced either with penicillin or with barium chloride were more resistant than the original to both penicillin and crystal violet. Youmans and Delves (1942), on the other hand, found small colony variants of *Staphylococcus aureus* no more resistant to barium chloride than were the strains from which they were derived. In the present work with *E. coli*, brief tests of resistance to heat and to crystal violet indicated that small colony variants were as susceptible to inhibition and killing by these agents as were original and reverted strains. Multiplication of small colony variants in concentrations of 2-methyl-1,4-naphthoquinone that did not permit growth of the normal colonial type indicates greater resistance to this particular agent. Their appearance in large numbers may have been due either to the multiplication of a few undetectable cells in this phase in the original inoculum or to injury of enzyme systems of normal cells of the inoculum, resulting in the development of the small colony variant. Actually, if the first of the foregoing explanations is accepted, the question of the reason for the original small colony variant is still open. The weight of the evidence appears to place the responsibility for the small colony variant on inhibition or destruction of enzyme systems necessary for normal growth by agents with some degree of antibacterial activity.

SUMMARY AND CONCLUSIONS

Stable small colony variants of four strains of *Escherichia coli* were produced in large numbers in synthetic medium containing low concentrations of 2-methyl-1,4-naphthoquinone. Reversion to forms indistinguishable from parent strains was achieved and occurred more readily in media enriched with glucose or tryptose or both. Inability of small colony variants to evolve gas from fermented sugars, weak acid production, and slow, meager growth all indicate that these small colony variants are cells of lowered metabolic activity, probably resulting from interference by the quinone with enzymes essential for normal development.

The possibility that they were present in small undetectable numbers in the original cultures and, being more resistant to the quinone, were able to multiply and make themselves apparent in quinone concentrations inhibitory to normal cell growth has not been ruled out, but it is actually not important in elucidating their origin.

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THE GROWTH OF BRUCELLA IN AERATED LIQUID CULTURES[1]

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Studies conducted on the immunochemistry of members of the genus *Brucella* required the growing of comparatively large amounts of these organisms in liquid culture medium. The production of a culture which would retain its full virulence over a period of six months was one of the major requirements of our work. Very little information on the growth requirements of *Brucella* was available in the literature from which to draw any conclusions on the practicability of culturing this group of microorganisms in deep columns of liquid media.

Although the genus *Brucella* is known to exhibit pseudoanaerobic growth, a property which is put to diagnostic use in the nitrate to nitrite reduction test, the yield of *Brucella* cells in statically grown flasks of tryptose broth is, in 48 to 72 hours at 37 C, very small except when very shallow layers of media are used.

An apparatus has been designed for producing, in routine fashion, 15-liter amounts of *Brucella suis* that are characterized by uniformity of yield, a virulence as high as that of the parent culture, and prolonged viability in the presence of protective substances such as dextrin.

EXPERIMENTAL

Apparatus. The apparatus to be described was constructed of parts which are standard equipment and readily available in most laboratories. It has the advantage that it may be handled as a unit, which fact facilitates the maintenance of sterility. Aseptic sampling of the contents is possible, with no limitation as to the size or frequency of the samples. From the standpoint of safety, which is of prime importance in studying pathogenic organisms, it can be reported that with this apparatus *Brucella* was produced successfully over a period of several months, and that none of the 35 laboratory workers exposed in the operation of the apparatus became infected in this period. As shown in figure 1, the apparatus is built around a 5-gallon pyrex carboy (6). The mouth of the carboy is tightly stoppered with a rubber stopper (8), which is wired to the neck of the carboy and sealed with de Khotinsky cement after sterilization. The various inlet and outlet tubes pass through the stopper. The air supply enters through the air filter (2), which is made up of a Kelly infusion bottle filled with nonabsorbent cotton. From the air filter the air flows through a pyrex stopcock (1) and then through a $2\frac{1}{2}$-by-$\frac{5}{8}$-inch Mandler filter (3), which serves to break up the air into very fine bubbles. The stream of bubbles rises rapidly and deflects against the layer of antifoam (7) to give a rotary circulation throughout the entire culture. The air leaves the carboy through the air filter (9). Inoculation is performed through the inoculating tube (4), which consists of a 100-ml volumetric pipette cut in half. The inoculating tube is stoppered with a long, tightly fitting, cotton-filled gauze plug when not in use. Sampling is done through the siphon (5), which ends in a pyrex filling attachment. The siphon is kept shut by a Hoffman clamp when not in use. The air filters (2) and (9) and the siphon (5) are tied to the neck of the carboy by means of iron wire and rubber bumpers, allowing the entire apparatus to be moved as a unit for operations such as sterilization. The connection of the rubber tubing to the Mandler filter, which is in the culture, is wired down with nichrome wire. All connections outside the carboy are wired down with no. 18 gauge copper wire. The stopcock (1) is kept closed and wired down during sterilization to prevent backing of the media into the air filter. Because of the prolonged high temperatures encountered during sterilization, ordinary stopcock greases will not retain their lubricating qualities. It has been found, however, that the recently developed silicones, as represented by Dow-Corning silicone, will successfully withstand sterilization with maintenance of lubricating consistency. Several alternate aerators have been successfully substituted for

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[1] Studies conducted at Camp Detrick, Frederick, Maryland, from September, 1944, to July, 1945.

[2] 1st Lt., C.W.S., and Major, Sn. C., respectively.

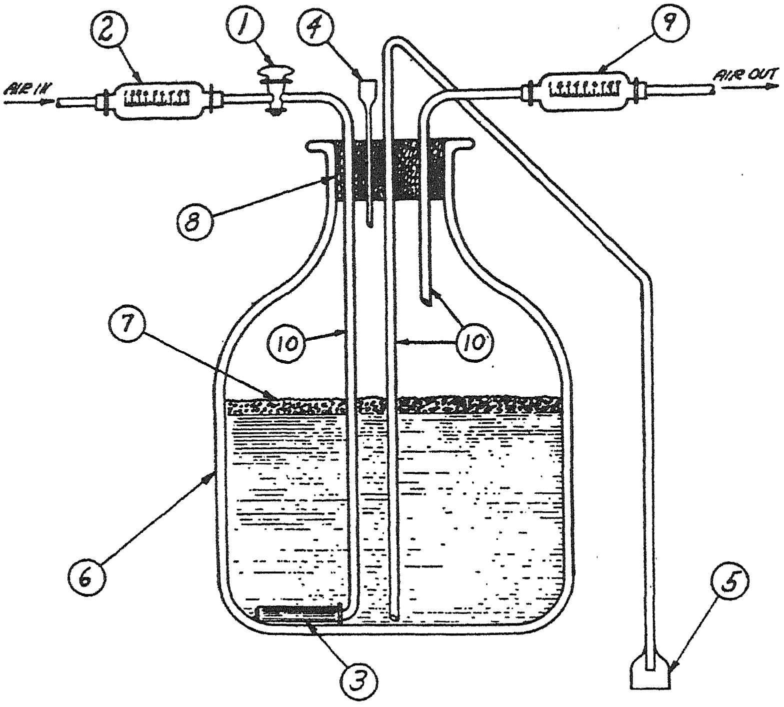

FIG. 1. APPARATUS FOR LARGE-SCALE LABORATORY AERATION

1. Stopcock.
2. Air filter.
3. Mandler filter.
4. Inoculating tube.
5. Sampling siphon.
6. Pyrex carboy.
7. Layer of antifoam.
8. Rubber stopper.
9. Air filter.
10. Rubber pressure tubing.

the Mandler filter. Among these may be mentioned the no. 10 porosity Selas filter, aloxite silicon carbide porous tubes (Carborundum Company, 1943), and porous carbon diffuser elements (National Carbon Company, 1943).

Production methods. A description of the production of a typical batch of *Brucella* will best illustrate the use of the apparatus. The stock culture was a transplant of *Brucella suis* strain 1772A obtained from Dr. I. F. Huddleson and maintained on tryptose agar slants. The liquid medium consisted of 2 per cent bacto tryptose, 0.5 per cent sodium chloride, all adjusted to a pH of 6.8 to 6.9. As an antifoam and air baffle a layer of pure lard was used. For 5 gallons of culture medium approximately 0.5 pounds of lard were used. For inoculum the surface growth was washed from a 48-hour tryptose agar slant of *Brucella suis* into 100 ml of tryptose saline solution (0.1 per cent bacto tryptose and 0.5 per cent sodium chloride). The temperature of incubation was 37 C. Aeration was maintained at a level of 1/15 volume of air per minute per volume of culture medium. Culture control consisted of determination of the numbers of viable organisms by the poured agar plate method using tryptose agar, morphological purity by gram stain, and colonial variation by observation of the surface growth on a tryptose agar streak plate illuminated by oblique reflected light. Virulence tests on cultures were performed by injecting graded doses into a series of guinea pigs and observing evidences of infection at autopsy 30 days after injection. The routine procedure at autopsy included culture of the ground livers and spleens of all animals and serological examination.

The medium and antifoam were poured into the aeration apparatus, and the entire unit was autoclaved for 90 minutes at 121 C. After about 20 hours of cooling to 37 C, the batch was left in the incubator for 24 hours to test the efficacy of the sterilization. If the holding period demonstrated that the medium was sterile, the batch was inoculated by pouring the washings from a 48-hour tryptose agar slant of *Brucella suis* through the inoculating tube. Aeration was initiated immediately after inoculation and maintained throughout the growth period at a rate of 1/15 volume of air per volume of culture per minute. Samples were removed periodically through the sampling siphon for plate count, gram stain, and observation for colonial variation. Maximal growth under these conditions was found at approximately 65 hours after inoculation, and a viable organism count of 1 to 2 $\times$ 10^{10} per ml was typical for *Brucella suis*. *Brucella abortus* and *Brucella melitensis* grown under the same conditions gave somewhat lower yields.

Growth of Brucella suis in aerated liquid culture. The response of *Brucella suis* to the growth conditions present in the apparatus just described is illustrated in table 1. The results presented in this table are representative of many of the early lots. The growth curve of the organisms in the aerated liquid medium resembles that obtained in flasks containing shallow layers of medium agitated vigorously during the growth period. A later series of 10 lots uniformly harvested after 65 hours of growth is presented in table 2. The increased yields shown in table 2 are more apparent than real, reflecting as they do increased familiarity with the production technique and more accurate assays of the viable count in the samples.

Aeration versus agitation. In deep culture, as represented by the 5-gallon carboy, it was questionable to what extent the aeration per se was the limiting factor, as compared with the agitation involved. In a recent review Becze and Liebmann (1944) summarize knowledge of the biochemical mechanism of aeration with special reference to yeast production. It is generally thought that aeration is important as a means of continually replenishing the oxygen supply, which is necessary because of the low solubility of oxygen in the culture medium, and because the sterilization process eliminates practically all the dissolved oxygen that was originally in the medium. It is also believed that aeration removes metabolic products from the surface of the cells and substitutes fresh nutrients for them. This function might, however, be just as well performed by mechanical agitation. In addition there is evidence that certain organisms, notably yeasts, will multiply in the absence of air, with agitation either by mechanical means or by an inert gas. In several experiments on a pilot plant scale Becze and Liebmann (1944) obtained considerable yields of compressed yeast in mashes through which carbon dioxide or nitrogen was passed instead of air. These yields were 3 to 4 times larger than those obtained in identical mashes in which no gas was distributed.

TABLE 1

The growth of Brucella suis (1772A) in a 5-gallon carboy culture apparatus

TIME	VIABLE ORGANISMS × 10^9 PER ML	
	Batch A	Batch B
hours		
24	1.5	1.9
48	4.1	12.0
72	9.5	12.0
96	4.2	4.1

TABLE 2

Yield of Brucella suis from a series of 15-liter batches harvested after 65-hour growth

BATCH NUMBER	VIABLE ORGANISMS × 10^9 PER ML
39	17
40	22
41	19.5
42	15
43	15
44	11
45	16
46	20
47	20.7
56	40

A batch has been run in which neither agitation nor aeration was used. Frequent sampling over a 72-hour period showed complete sterility, and visual inspection showed a clear, transparent medium, indicating that the inoculum had not even diffused to the bottom of the culture apparatus where the inlet of the sampling siphon is located. Obviously then, agitation or aeration, or both, are required. To separate these factors two types of experimentally agitated batches were grown. In one batch nitrogen was used to agitate the culture medium. In the other batch agitation was obtained by the continual rotation of the medium by means of a steel propeller As a control experiment, a batch was produced using atmospheric air for aeration. With both the nitrogen and the air control the volume of gas passed through the culture medium was the same (1/15 volume gas per volume of culture medium per minute). The results are recorded in table 3. It is obvious from these experiments that agitation, whether by an inert gas or mechanical means, is not sufficient to support multiplication of *Brucella suis.*

TABLE 3

Comparison of growth of Brucella suis with agitation by nitrogen, mechanical agitation, and aeration

TIME	VIABLE ORGANISMS × 10^9 PER ML		
	Nitrogen 1/15 volume per min	Propeller agitation	Aeration 1/15 volume per min
hours			
24	0.3	0.1	0.88
48	0.025	0.2	8.4
65		0.2	11.0
72		0.2	8.6
96		0.2	5.3

The growth under aeration, as shown in table 3, was found to be typical, with maximal yields at approximately 65 hours after inoculation, so that routine production was instituted using a 65- to 72-hour harvesting period. Yields as high as 4 × 10^{10} viable organisms per ml have been obtained, with an average of 1.5 × 10^{10} viable organisms per ml. Although these figures apply to *Brucella suis*, similarly aerated batches of *Brucella abortus* and *Brucella melitensis* have been run with somewhat lower yields.

Carbon dioxide requirements. In addition to their oxygen requirements the species of *Brucella* seem also to be sensitive to the carbon dioxide content of their environment. This is reported to be especially true in the case of *Brucella abortus*, with which an increased carbon dioxide tension of about 10 per cent over that of atmospheric air is necessary for primary isolation, and is beneficial to growth in liquid medium. In the case of *Brucella suis* growth is reported as much more rapid when liquid medium is incubated in air enriched with 10 per cent by volume carbon dioxide (Huddleson, 1943). To determine whether the presence of 10 per cent carbon dioxide by volume added to the air used in aeration would have any beneficial effect upon the mass growth of *Brucella*, a batch was produced in which air plus 10 per cent added carbon dioxide was used for aeration. As a control a batch aerated with ordinary atmospheric air was used. Reference to table 4 will show that there is no significant difference in the growth of *Brucella suis* under these two conditions. The presence of added carbon dioxide does not seem necessary nor beneficial.

Dissociation. The striking increase in yields of *Brucella* in aerated liquid media that was routinely obtained after growth in the apparatus described herein made it imperative to determine whether dissociation of the organism occurred during the culture cycle. The two characteristics that were examined as accurately as possible were colonial form and minimal infective dose for the guinea pig.

In examinations of many subcultures on tryptose agar from each aeration culture, using oblique reflected light, no significant colonial dissociation was detected that conformed to the variants described by Huddleson (1943) and Henry (1933) for *Brucella abortus*. The selection of many smooth colonies for the determination of the minimal subcutaneous infective dose in guinea pigs failed to show evidence of dissociation. Simultaneous determinations of catalase activity by the method of Huddleson (1943) on similar cultures also failed to reveal evidence of variation as a result of this method of culture.

TABLE 4

Comparison of the growth of Brucella suis in cultures aerated with air alone and with air plus added carbon dioxide

TIME	VIABLE ORGANISMS × 10^9 PER ML	
	10 Per Cent carbon dioxide added	Air alone
hours		
24	2.0	0.9
48	6.5	8.4
56	7.2	—
65	9.6	11.0
72	8.0	8.6
96	6.5	5.3

Virulence. More importance, however, was attached to virulence tests on the liquid aerated culture than to any other determination of its conformity to agar-grown *Brucella.* Routine determinations of virulence were performed in two ways. One was to inject decimal dilutions of culture subcutaneously into guinea pigs. Comparison was made with the same strain grown on tryptose agar, the latter being considered the "standard" culture. Lots of 10 guinea pigs were used for each dose of the "standard" and aerated liquid cultures. Thirty days following injection the animals were sacrificed and examined for the presence of typical lesions in the liver, spleen, and regional lymph nodes. Cultures of these organs were made, and the isolation of the organism from one or more of these tissues was accepted as the final criterion of infection. The results of many such titrations indicated that the agar slant and the aerated liquid culture methods produced organisms of equal virulence. The minimal infective dose for 50 per cent of the animals was of the order of 5 organisms when injected subcutaneously.

One of the requirements of this study was that the virulence of the organisms remain at its maximum by the respiratory route of infection as well as by the subcutaneous or intradermal routes. The apparatus and general methods used to study respiratory *Brucella* infection will be reported elsewhere. The results of the exposure of groups of guinea pigs to airborne *Brucella suis* prepared on agar media and in the liquid aeration apparatus are presented in table 5. It will be seen that culture of the organism in the aeration apparatus caused no loss of virulence as compared with that of the tryptose-agar-grown "standard."

TABLE 5

The influence of the culture process on respiratory virulence of Brucella suis

TRIAL	CULTURE METHOD	ESTIMATED NUMBER OF ORGANISMS PER ANIMAL	GUINEA PIGS USED	PER CENT INFECTION
1	Tryptose agar (standard)	48	40	66
	Aerated liquid Tryptose broth	55	40	61
2	Tryptose agar (standard)	53	20	31
	Aerated liquid Tryptose broth	44	20	50

SUMMARY

An apparatus suitable for the production of pathogenic microorganisms in 15-liter lots is described. A study of the growth of *Brucella suis* in the apparatus is presented.

The importance of aeration as distinguished from mechanical agitation has been demonstrated.

Enrichment of the air with 10 per cent by volume carbon dioxide is shown to be neither necessary nor beneficial to 15-liter volumes of aerated liquid-grown cultures of *Brucella suis*.

Expansion of production batches from test tube agar slants to 15-liter liquid lots was effected without the occurrence of dissociation, an accomplishment that was evidenced by lack of change in colony morphology and in minimal infective doses for guinea pigs.

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FURTHER STUDIES ON ONE ANAEROGENIC PARACOLON ORGANISM, TYPE 29911

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Interest in anaerogenic paracolon, type 29911, was aroused when the first strains were isolated in 1938. Concern for these cultures diminished greatly when it was found that they were a heterologous group serologically (Stuart, Wheeler, *et al.*, 1943; Rustigian and Stuart, 1945). During the period from 1938 to the summer of 1945 about 150 strains of type 29911 were identified, primarily on the basis of biochemical reactions, and discarded.

In the summer of 1945 it was found that three cultures, "*B. wakefield*," described by Berger (1945) as members of the *paradysenteriae* group possessed the biochemical reactions of type 29911 (Wheeler and Stuart, 1946). In view of this and because the *B. wakefield* cultures agglutinated to a 5,120 titer in an antiserum prepared from a type 29911 culture (EEB), it was decided to reexamine the 29911 group. Cultures were collected from Providence and vicinity, particularly the Rhode Island Hospital, and from the Connecticut and Florida State Public Health Laboratories. In about 4 months 109 cultures of type 29911, or closely resembling 29911, were obtained from the specified sources with an occasional culture from elsewhere. Sixty-three or 57 per cent of the cultures came from Florida.

Biochemical reactions. Table 1 shows the biochemical reactions of type 29911 and related cultures. In the first group are recorded the reactions considered "typical" of type 29911, though an occasional strain may differ in one reaction such as fermentation of arabinose or inulin, or failure to ferment maltose or glycerol, or to produce indole. Other groups shown in table 1 differ from typical 29911 strains in two or more reactions. One or more cultures of group 1 were antigenically related to certain cultures in each of the other five groups. For the foregoing reasons, and because a strain may vary in one or two reactions on repeated tests, the number of cultures in each group are not recorded in table 1. Approximately 75 of the 109 cultures fell in group 1, and there were 19 strains in group 6, which will be discussed below.

Typical type 29911 cultures produce acid or acid and a small bubble of gas rapidly in glucose. Sucrose is fermented without gas in 5 to 30 days; acid first appears in the vial while the medium outside is alkaline; eventually the entire medium becomes moderately acid and the indicator (bromcresol purple) is reduced. Maltose is attacked like sucrose but more slowly. Adonitol is fermented rapidly and glycerol slowly. Lactose, mannitol, arabinose, xylose, rhamnose, galactose, cellobiose, trehalose, raffinose, inulin, sorbitol, dulcitol, and inositol ordinarily are not fermented. Indole is produced but not acetylmethylcarbinol.

Growth on sodium citrate occurs more rapidly at room temperature and may be negative at 37 C. Hydrogen sulfide production in lead acetate agar was first recorded as negative, but positive results were obtained with tryptone broth and lead acetate paper. Trimethylamine oxide is reduced, but the reaction is occasionally weak. Many cultures reported nonmotile proved to be motile at room temperature. Of nine nonmotile cultures from Florida, only three are still nonmotile after serial transplants in semisolid agar for 5 months.

The strains in group 6 (table 1) were isolated from 19 individuals who were the only cases studied among approximately 80 involved in a gastroenteritis outbreak caused by chicken salad (Dr. H. Plass, personal communication). Antiserums were prepared from 2 cultures. Each of the 19 strains agglutinated to titer in these antiserums and upon adsorption completely removed the homologous agglutinins. Cultures with the same biochemical reactions were isolated from the chicken salad but were not available for the present work.

TABLE 1

Biochemical reactions of type 29911 (group 1) and related cultures

GROUP	GLUCOSE	SUCROSE	MALTOSE	MANNITOL	GALACTOSE	CELLOBIOSE	TREHALOSE	INULIN	GLYCEROL	ADONITOL	SORBITOL	INOSITOL	LACTOSE SALICIN ARABINOSE XYLOSE RHAMNOSE RAFFINOSE DULCITOL	INDOLE CITRATE T.M.A. MOTILITY	V-P UREA	HYDROGEN SULFIDE
1	A	As	As	—	—	—	—	—	As	A	—	—	—	+	—	+w
2	A	As	As	—	A	—	—	—	As	—	—	—	—	+	—	+m
3	A	As	As	—	A	—	—	As	As	—	—	—	—	+	—	+w
4	A	As	—	—	A	As	—	—	A	—	As	A	—	+	—	+m
5	A	As	—	As	A	—	A	—	As	—	As	A	—	+	—	+m
6	A	As	—	As	A	—	A	—	A	A	As	A	—	+	—	+m

A = acid (bubble of gas may be formed in glucose); As = delayed acid; + = positive reaction; +w = weakly positive; +m = moderately positive; T.M.A. = trimethylamine oxide.

Colonial variants. On standard agar slants when the inoculum covered the surface of the slant, a relatively thin film of growth developed. By transmitted light the growth was translucent and bluish green. This is characteristic of 29911 and related types. An occasional culture developed areas of heavy, white, opaque secondary growth, which appeared like a contamination. Plating from a presumably uncontaminated area of such a slant on EMB agar and SS agar yielded only normal 29911 colonies. Plating from an opaque area on the same medium gave no growth on SS agar. On EMB agar no growth occurred except occasionally when several colorless, small (1 mm or less) colonies appeared on a plate. Since smears from such colonies showed only gram-negative, nonspore-forming rods, inoculums from the same area of the slant culture were streaked on nutrient agar plates. Three types of colonies developed. (1) Flat entire colonies approximately 5 mm in diameter. These colonies were butyrous and by transmitted light were translucent and bluish green. In broth at room temperature, growth was uniformly turbid and composed of individual short rods 3 to 5 μ long and actively motile. This was called the smooth or S form. (2) Somewhat raised colonies about 8 to 10 mm in diameter with erose edges. By transmitted light they appeared filamentous in structure and highly iridescent, with a somewhat mucoid consistency. In broth, growth was very granular and composed of skeins of long rods, which were sluggishly motile or nonmotile. This was called the rough-mucoid or R-M form. (3) Convex colonies, white and opaque, with a marked mucoid consistency. After 3 to 5 days the tops of such colonies became depressed with the subsequent formation of one or more papillae. Growth in broth was mucoid and composed of threads of small motile rods. This form was recorded as mucoid or M.

Straight needle inoculums from smooth broth cultures resulted in good uniform growth on EMB, SS, and desoxycholate citrate agars. Large loop inoculums from R-M or M broth cultures yielded, for the most part, no growth on EMB agar or occasionally the small colonies described above; on SS and desoxycholate citrate agars no growth occurred. Such variants, for the most part, reverted to the parent type quite readily.

These colonial variants with their inability to grow on certain mediums are similar to the colonial variants of *Shigella sonnei*, which, according to the findings of Wheeler and Mickle (1945), failed to grow on SS and desoxycholate citrate agars. It is evident that if variants of *S. sonnei* and type 29911 which fail to grow on mediums used to isolate enteric organisms occur in nature and are pathogenic, sporadic cases and epidemics could occur in which ordinary technique would fail to ascertain the etiological agent.

Swarming characteristics. Stuart, van Stratum, and Rustigian (1945) noted that the growth of some strains of 29911 on agar slants spread upwards for several millimeters from the water of expression. To determine whether 29911 cultures could be induced to swarm, 63 actively motile strains, representing all groups in table 1, were transferred daily for 1 week in tubed semisolid agar, then onto 1 per cent agar slants. Spreading on such slants increased with the number of transplants. When the growth of a strain covered from 75 to 100 per cent of the slant surface (3 to 12 transplants), an inoculum from the uppermost portion of spread was transferred to the center of a 1 per cent agar plate (100 mm). After 48 hours at room temperature an inoculum from the greatest margin of spread was transferred to another plate.

The growth of the 63 cultures tested completely covered the agar surface of the plate in 48 hours. One culture did so on the first transfer to a plate, but others required from 3 to 8 platings. With some cultures the growth was definitely spreading, a more or less continuous wave of growth outward from the site of inoculation, with few if any branches and with only very minor variations in the thickness of growth. With some cultures the growth was definitely swarming, bizarre branches of growth, straight, curved, or even looped, emanating from the site of inoculation with islands of relatively thick growth in a thin film. Still other strains exhibited a combination of spreading and swarming. Rustigian and Stuart (1945) found that *Proteus rettgeri* exhibited swarming tendencies, but only an occasional culture covered the surface of a 1 per cent agar plate. Twenty four *P. rettgeri* strains treated as described for 29911 completely swarmed over the surface of an agar plate after one to five platings.

Urease production. Type 29911 cultures fail to give strong reactions in standard urea medium even after 28 days' incubation. Of approximately 300 cultures tested to date, only 3 exceptions to this rule were encountered. One culture received from Lt. M. Fisher, Army General Hospital, Denver, Colorado, which was strongly positive when isolated, subsequently attacked urea weakly. Plating this strain on urea agar medium resulted in areas on the plate ranging from red to colorless. Serial platings and selections on this medium yielded a urea-positive variant of this culture that has remained stable for more than 4 months.

A *P. rettgeri* strain requiring 3 days for a positive urea reaction was also received from Lt. Fisher. On the first plating on urea agar this culture produced red, pink, and colorless areas. Despite plating and selection as in the previous case, the urease-producing property of this strain was completely lost. In the first culture urease production was fixed on a type normally negative, whereas with the second culture the same property was lost in a normally positive species. These gain and loss variations illustrate the difficulty of assigning absolutely fixed biochemical properties to any species.

Two cultures from Florida were urea-positive in 18 hours. As the number of subcultures from the original culture increased, the time required to give a positive reaction increased until this property was completely lost. No attempt was made to fix urease production in these two strains by selection.

Antigenic interrelationships. An exhaustive study of the interrelationships of type 29911 cultures was not made in this phase of the work. However, all such strains and related types were tested for agglutination in three type 29911 antiserums, EEB, 2634, and 16112, with homologous titers of 81,920, 20,480, and 10,240, respectively. Certain cultures were also tested in antiserums prepared against nonmotile and motile variants of the *B. wakefield* strains. Saline suspensions of living organisms were used as antigens in agglutination tests except when otherwise specified. A limited number of adsorption tests were made, and these were also done with living antigens.

Contrary to expectation, a relatively marked continuity of antigens was found in the group. Of 109 cultures tested, only 26 per cent failed to agglutinate in any antiserum. The remaining cultures agglutinated to titers ranging from 80 to the homologous titer of one or more of the 3 antiserums. Apart from the facts that (1) cultures used in this phase of the work were isolated and tested in a period of approximately 4 months, (2) cultures came from relatively restricted areas, (3) longer incubation was used in agglutination tests, and (4) one strain, EEB, used to produce an antiserum possessed unusually broad antigens, it is difficult to reconcile this antigenic continuity with the antigenic heterogeneity observed in the early work.

In the present work where cross reactions were common, it was soon found that incubating tests for 2 hours at 37 C followed by overnight at 55 C was not wholly satisfactory. Many minor and some major cross reactions that were positive at 37 C were completely negative at 55 C. Incubation for 2 hours at 37 C followed by overnight at 2 C gave consistently higher titers and a greater number of cross reactions. Low temperature incubation presented one difficulty which, except for *Proteus*, is not ordinarily encountered among the *Enterobacteriaceae*. Cultures giving strong (++++) floccular or granular agglutination at 37 C gave at 2 C only fine granular reactions that were very difficult to read. Moreover, many tests which were negative after 2 hours at 37 C gave weak to indeterminate reactions at 2 C. These same tests when incubated for 6 hours at 37 C yielded titers of 320 to 10,240 in one or another of the three antiserums. Such tests showed fine granular agglutination at 37 C; the degree of agglutination was not altered at 2 C and was completely reversed at 55 C.

Thirteen cultures agglutinated to titer in EEB antiserum. Upon adsorption, nine of these strains reduced the homologous titer from 81,920 to 640, but four failed to alter the homologous titer. Eighteen cultures agglutinated to titer in antiserum 16112. Upon adsorption, four cultures reduced the homologous titer from 10,240 to 320, but the remaining cultures failed to reduce the homologous titer. Three cultures agglutinating to high titer were used to adsorb antiserum 2634, but the homologous titer was not altered. Despite the antigenic continuity of type 29911 cultures found in this phase of the work, strains antigenically identical to those used to produce the three antiserums were by no means common.

The somatic antigens of several type 29911 cultures appeared to be destroyed by boiling and by treatment with alcohol. This was particularly true of strain EEB. Normal EEB antigen in its own antiserum after 2 hours at 37 C gave soft, flocculent H agglutination to a titer of 81,920, whereas after 18 hours at 55 C granular O reactions were obtained to 40,960. Moreover, several strains showed only granular agglutination to titers of 10,240 to 20,480 in EEB antiserum, and one strain, nonmotile when isolated, agglutinated to 10,240. From this it is evident that the O titer of EEB antiserum was at least 10,240. Nevertheless, EEB antigen heated for as little as 5 minutes after reaching a temperature of 65 C, or treated with absolute alcohol for 5 minutes at room temperature, gave a titer of only 640 in its homologous antiserum. More rigorous heating, including 2 hours at 121 C, did not destroy this residual antigen. All cultures agglutinating to titer in EEB antiserum reacted the same.

Several type 29911 cultures including EEB agglutinated in *B. wakefield* antiserums to titers ranging from 320 to 10,240. (Bridges and Taylor, 1946, concluded that the "wakefield strains" were members of the paracolon rather than of the Flexner group of organisms.) Moreover, 2 cultures described by Sachs as *Shigella* type B105 possessed the biochemical characteristics of typical 29911. One of these cultures agglutinated in 16112 antiserum to a titer of 5,120, and in *B. wakefield* antiserum 2624H to 160; and the other agglutinated in 2624H antiserum to 10,240, and in 16112 antiserum to 160. Another Sachs culture, B81, with the same biochemical reactions as B105 failed to agglutinate in 29911 antiserums except for a small cross (160) in 2624H antiserum. It was previously pointed out (Wheeler and Stuart, 1946) that two Sachs types, B81 and B105, do not belong in the *Shigella* group. Biochemically these strains appear as typical 29911 cultures, fermenting maltose after prolonged incubation. The finding of antigens in common with other type 29911 cultures confirms their classification in this group.

Antigenic relationship to Proteus. Fifty-five type 29911 cultures, including one or more strains from each group (table 1), were tested in a total of 16 antiserums prepared against *P. vulgaris*, *P. mirabilis*, *P. morganii*, and *P. rettgeri*. Only 3 cultures failed to agglutinate in any antiserum, whereas each antiserum agglutinated from 3 to 29 cultures. For the most part, cultures agglutinated to titers of 40 to 2,560, but an occasional strain reacted to titers of 5,120 to 10,240 or higher. One type 29911 strain agglutinated to titer (40,960) in one *P. vulgaris* antiserum, a second culture to titer (20,480) in another *P. vulgaris* antiserum, and a third culture to titer (20,480) in a *P. morganii* antiserum. Upon adsorption, the titer of one *P. vulgaris* antiserum was reduced from 40,960 to 1,280, the titer of the other *P. vulgaris* antiserum was not altered, whereas all agglutinins were removed from the *P. morganii* antiserum.

Forty-eight *Proteus* cultures, including all four species mentioned above, were tested in four type 29911 antiserums. Eleven cultures failed to agglutinate in any antiserum, but each of the four antiserums agglutinated from 14 to 22 *Proteus* cultures, for the most part, to titers ranging from 40 to 2,560. One *P. vulgaris*, one *P. morganii*, and two *P. rettgeri* cultures agglutinated to sufficiently high titers to warrant adsorption, but in no antiserum was the homologous titer reduced. It is evident that an occasional type 29911 and *Proteus* culture may possess major antigens in common or may be closely related antigenically.

DISCUSSION

Type 29911 cultures present an interesting and important problem in taxonomy. Included in this group are cultures which were previously designated as an anaerogenic paracolon type (Stuart, Wheeler, *et al.*, 1943), cultures formerly classified as *B. wakefield* related to the Flexner dysentery organism (Berger, 1945), and cultures described as mannitol-negative types of *Shigella*, Sachs types B81 and B105 (Sachs, 1943). The present study indicates that type 29911 closely resembles *Proteus* species in: (1) their biochemical and IMViC reactions, (2) their ability to swarm when properly conditioned, (3) their reaction to urea, an occasional strain being weakly positive and an exceptional strain strongly positive in urea medium, and (4) their many minor and occasional major antigens in common with *Proteus*. To include these organisms in the genus *Proteus*, however, calls for a definite extension of the limitations of the genus which does not seem advisable at the present time. To create new genera and to raise certain genera to family rank in the *Enterobacteriaceae* as has been suggested (Fulton, 1943; Waldee, 1945; and others) would simplify the immediate problem but could easily confuse future considerations. The position of type 29911 strains appears to be intermediate between the *Proteus* and the *Shigella* groups, and at present they can be classified only as transitional forms between these two groups and given neither genus nor species rank.

Aside from the theoretical importance of type 29911 as an intermediate group between recognized genera, it has practical importance in enteric bacteriology. Considerable evidence has been accumulated to show that type 29911 organisms cause gastroenteritis and diarrhea. Berger's strains of *B. wakefield* came from an outbreak of diarrhea among children, from a case of acute gastroenteritis, and from a case of recurrent diarrhea. Sachs reported 11 isolations of type B81 or B105 from cases of diarrhea and failed to find these organisms in examinations of over 15,000 specimens from food handlers, etc. Our own experience has shown that, with few exceptions, type 29911 cultures were isolated from gastroenteritis patients. Some of these patients were hospitalized. From over 300 normal individuals, students, food handlers, nurses, etc., only one strain of 29911 was isolated, and this carrier gave a history of diarrhea a week previous to the isolation. A number of cases have shown recurrence. One individual in about one year had three separate attacks of gastroenteritis. Type 29911 was isolated from the first attack, but the culture was discarded. The same type was recovered from the second and used to produce EEB antiserum. The same type (EEB_2) was isolated from the third attack but was antigenically unrelated to culture EEB. On the other hand, type 29911 cultures were frequently encountered in specimens from food handlers by the Florida State Public Health Laboratories (Galton, personal communication). Isolations of 20 to 30 cultures per month have been made by that laboratory during the past year, and histories of these cases should provide extremely valuable information concerning the significance and public health importance of type 29911 strains. (A report on the isolation, distribution, and public health aspect of 29911 cultures will come in the near future from the Florida State Board of Health Laboratories.) Serological work so far done within the 29911 group shows that many sero-types are included. Further work directed toward identification of sero-types in the group, particularly if correlated with clinical history, should provide information of marked public health value.

ACKNOWLEDGMENT

We are indebted to Mrs. Mildred Galton, Florida State Board of Public Health Laboratories, whose co-operation contributed greatly to the scope of this work.

CONCLUSIONS

One hundred and nine cultures of anaerogenic paracolon, type 29911 and related cultures, were found to be biochemically and serologically heterogeneous.

These organisms resemble *Proteus* in many characteristics but appear to be a transitional group between *Proteus* and *Shigella*.

Some cultures appear quite definitely to have been the etiological agent in gastroenteritis.[1]

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[1] After completing this manuscript, 11 cultures with the biochemical reactions of type 29911 were received from Dr. de Assis, Rio de Janeiro, Brazil, and 3 cultures from Capt. Rustigian, Sn.C., U.S.A., isolated from native Italians. Each of the 14 cultures agglutinated to titers ranging from 320 to the homologous titer in one or more of 14 antiserums

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prepared from 29911 strains isolated in the eastern part of the United States. Ten cultures agglutinated to sufficiently high titers to warrant adsorptions. One culture from Brazil completely removed agglutinins from one antiserum, but the remaining 9 cultures failed to reduce the homologous titer of the antiserums involved.

THE ADHESION OF RABBIT PLATELETS TO BACTERIA[1]

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The phenomenon of adhesion between cellular elements of the blood and bacteria or other foreign particles has many applications; however, we are particularly concerned with the formation of "infective" thrombi, or bacteria-platelet thrombi, and the role of these thrombi in immunity and disease. Early in this century Aynaud (1911), Roskam (1921, 1927), Govaerts (1921*a*, 1921*b*), and Delrez and Govaerts (1918) investigated the importance of platelets in the body defenses against bacterial invasion. A review of the relevant literature will be found in the comprehensive monograph by Tocantins (1938). These workers showed that certain bacteria adhere to platelets, whereas others do not, and postulated the "antixenic" function of the blood platelet. In the majority of their studies the rabbit was the experimental animal. Examinations of guinea pigs and dogs were much less extensive, but thorough enough to indicate that platelets from different species may vary in their affinity for bacteria and particulate matter. Little attention has been given to this point, and no satisfactory explanation can be given at present.

Delrez and Govaerts (1918) did not think that specific bacterial antibody was responsible for the adhesiveness of the platelets to bacteria, inasmuch as type-specific pneumococcal antiserum caused the pneumococci to agglutinate *in vivo* in the rabbit, but did not cause them to adhere to the platelets. Govaerts (1921*b*) suggested that the virulence of an organism determined its platelet adhesiveness because unsensitized pneumococci, which did not adhere to rabbit platelets, produce a septicemia in this animal. Aynaud (1911) did not believe that virulence was the determining factor, since he observed that both pathogenic and nonpathogenic bacteria may stick to platelets. Bull and McKee (1922) cast doubt on the significance of platelets in body defense by demonstrating that bacteria are removed from the circulating blood of a deplatelized rabbit as rapidly as from that of a normal rabbit.

In previous investigations we have studied the factors which affect the agglutinability of *isolated* platelets of humans, dogs, and rabbits (Copley and Houlihan, 1946). This report represents an attempt to apply this knowledge to a study of the mechanism responsible for the adhesion of bacteria to platelets.

METHODS

Preparation of blood-bacteria samples. Infusion broth cultures were washed twice with isotonic saline containing 0.2 per cent formaldehyde and adjusted to a

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concentration of 1,000,000 cells per mm^3 by counting in the Petroff-Hausser chamber. Rabbit blood was taken from the heart into a syringe containing 3.8 per cent sodium citrate, using 1 part of citrate solution to 4 parts of blood. This citrated blood was thoroughly mixed and distributed in glass tubes in the icebox. One sample was immediately rotated at 1 rpm for 30 minutes at 37 C and a platelet count made using a method described previously (Houlihan and Copley, 1946).

The citrated blood was diluted 1:10 in 3.8 per cent sodium citrate, then 1:2 in brilliant cresyl blue dye, and examined in the Petroff-Hausser counting chamber using an oil-immersion objective and 10 × ocular. The sediment from 1 ml of stock bacterial suspension was resuspended in 1 ml of chilled citrated blood and rotated for 30 minutes as above. The platelets were counted, a platelet clump being recorded as one platelet. At the same time, the size and number of platelet clumps containing bacteria were noted. This process was repeated with several bacterial strains. Upon completion of the tests, another control platelet count was made on the citrated blood which had been stored in the icebox. One such experiment required an average of 3.5 hours for completion.

Methods for calculating adhesiveness of platelets and bacteria. The time and temperature of rotation adopted in this study were based on evidence obtained in a previous investigation of platelet counts in rotated citrated and heparinized rabbit bloods (Houlihan and Copley, 1946). In that report it was demonstrated that platelets are stable in citrated rabbit blood which has been rotated for 30 minutes at 37 C following storage in the icebox for at least 3.5 to 4.0 hours (figure 1, no. 2). This finding thus made it possible to test several bacterial strains in the *same* blood sample. Heparinized blood could not be used in similar tests because the platelets clumped shortly after the withdrawal of blood (figure 1, no. 6).

The extent of the decrease in the platelet count which occurred in the rotated blood-bacteria samples was determined for two reasons: (1) to check the accuracy of the index used to denote the degree of adhesion of bacteria and platelets; and (2) to find out whether or not soluble bacterial substances might cause agglutination of the platelets. The platelet count was assumed to decrease progressively in stored and rotated citrated blood (Houlihan and Copley, 1946), and the degree of platelet agglutination produced by specific bacteria was determined in the following manner: the difference in the control platelet counts at the beginning and the end of each complete experiment was divided by the total elapsed time; thus the expected count could be calculated for any unit of time. Since the time of the testing of each organism was known, a comparison of the actual platelet count with the count calculated as above yielded the platelet count decrease produced by that particular organism. This method is, of course, subject to a wide margin of error, inasmuch as the platelet count may fluctuate during the course of the experiment.

The accuracy of the two Petroff-Hausser chambers used was determined by using washed red cells and one pipette. The standard error was 5.4 in one chamber and 7.4 in the other; t, calculated from Fisher's tables (1934), was 0.1, indicating that there was no significant difference between the chambers.

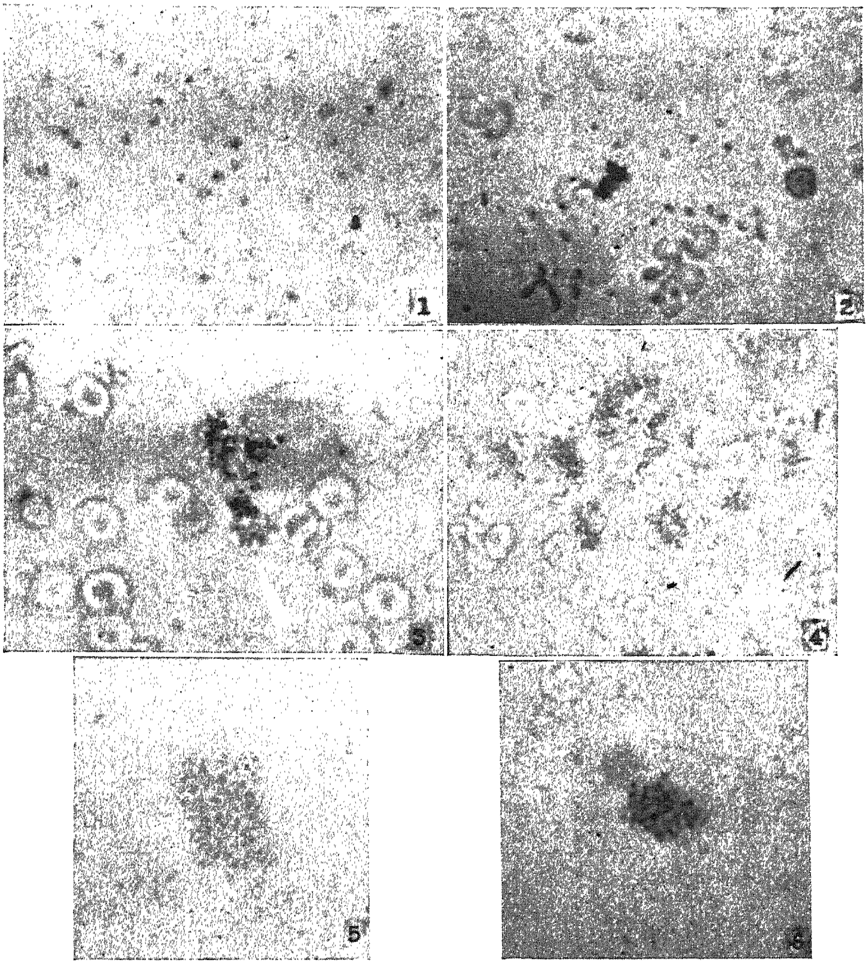

FIG. 1

All preparations were stained with brilliant cresyl blue dye and photographed in a fluid state at a magnification of 900 ×.

No. 1. A saline suspension of platelets isolated from citrated rabbit blood. The platelets are dispersed and show no tendency to agglutinate. The dark-stained granules in the cytoplasm are distinct.

No. 2. A mixture of citrated rabbit blood and *Streptococcus salivarius* which has been rotated. The platelets are stable and are not attracted to the dark-staining clump of bacteria.

No. 3. Mixture of citrated rabbit blood and *Staphylococcus aureus* which has been rotated. Numerous cocci are enmeshed in a platelet clump (indicated by arrow).

No. 4. Mixture of rotated citrated rabbit blood and *Streptococcus pyogenes*, showing several clumps of platelets containing or adhering to short and long chains of cocci.

No. 5. A platelet clump which has been produced by centrifuging a mixture of isolated rabbit platelets and human plasma. The individual cells in the clump have retained the granules and apparently have not fused.

No. 6. Platelet clumping in rabbit blood containing 1 mg sodium heparin per ml.

During the counting of platelets, which was done within a limited time, the number and size of platelet clumps were graded as small, medium, or large, if they were comprised of 2 to 3, 4 to 7, or more than 7 platelets, respectively.

The total numbers of small, medium, and large mixed clumps were multiplied by average factors of 1, 2.5, and 5, respectively, to obtain an *index* of adhesion of platelets to bacteria. Although this method of determining the index of adhesion is satisfactory when the actual number of platelets in a clump can be determined, it is not adequate when large clumps, some covering several microscopic fields, are seen. These instances are noted in the text.

TABLE 1

Comparison of platelet count decreases in mixtures of bacteria and whole blood with agglutinin titers

BACTERIAL STRAINS	D AND T*	NORMAL RABBITS			RABBITS IMMUNIZED WITH			RANGE	AVERAGE
		No. 1	No. 7	No. 10	*S. faecalis* 1430	*S. mitis* 1432	*S. salivarius* 1431		
					No. 90	No. 97	No. 98		
Streptococcus salivarius 1431	D	25	23	16	15	3	28	3–28	18
	T	1/64	>1/64	1/64	1/16	1/32	>1/128		
Streptococcus mitis 1432	D	62	23	19	29	71	41	19–71	41
	T	<1/8	<1/8	<1/8	1/16	1/16	<1/16		
Streptococcus bovis 1425	D	87	78	78	89	72	83	72–89	81
	T	1/64	1/64	>1/64	1/32	<1/16	1/64		
Streptococcus pyogenes 1408	D	90	84	74	63	31	63	31–90	67.5
	T	1/32	<1/8	1/32	>1/64	1/64	>1/64		
Staphylococcus aureus 1307	D	53	49	64	68	64	69	49–69	61
	T	1/32	1/32	1/128	>1/64	>1/64	>1/64		
Staphylococcus citreus 1302	D	40	24	13	37	48	67	13–67	38
	T	<1/8	<1/8	<1/8	<1/16	<1/16	<1/16		
Streptococcus faecalis 1435	D	77	78	76	80	68	96	68–96	79
	T	1/16	<1/16	<1/16	<1/16	<1/16	<1/16		
Streptococcus faecalis 1430	D	95	71	69	72	71	99	71–99	79.5
	T	<1/8	1/64	<1/8	1/256	1/16	<1/16		
Streptococcus salivarius St5E	D	44	21	14	33	5	46	5–46	27
	T	1/32	1/256	1/16	1/16	1/32	<1/16		
Streptococcus salivarius St25D	D	35	21	20	25	9	14	9–35	21
	T	1/32	1/64	1/16	<1/16	<1/16	<1/16		

* D = Platelet count decreases in per cent. T = Bacterial agglutinin titers.

EXPERIMENTAL AND RESULTS

Rotation of bacteria in whole citrated blood. Staphylococcus aureus, Staphylococcus citreus, Streptococcus bovis, Streptococcus pyogenes (group A), *Streptococcus* *mitis*, *Streptococcus salivarius* (3 strains), and *Streptococcus faecalis* (2 strains) were each tested in 12 whole blood samples from 6 rabbits. The data on the percentage of platelet count decrease and the index of platelet adhesion produced by these bacteria are presented in tables 1 and 2 and figure 2. Since the platelet count decrease in the blood-bacteria samples affords indirect evidence of the degree of the adhesion of platelet clumps to bacteria, these data will be compared with the indices of adhesion.

The tests with *S. salivarius* showed that the platelets do not agglutinate or adhere to this organism, since the index of adhesion was 0 in 14 of 18 tests using the three strains. The average indices for the three strains were 1.5, 0.2, and 0.3. The average platelet count decreases produced by the three strains of 18, 27, and 21 per cent were not considered to be indicative of platelet-bacteria adhesion.

TABLE 2

Adhesiveness of platelets and bacteria in normal and immune rabbit blood

BACTERIA	NORMAL RABBITS			RABBITS IMMUNIZED WITH			RANGE	AVERAGE
	No. 1	No. 7	No. 10	*S. faecalis* 1430	*S. mitis* 1432	*S. salivarius* 1431		
				No. 90	No. 97	No. 98		
Streptococcus salivarius 1431	1	8	0	0	0	0	0–8	1.5
Streptococcus mitis 1432	13	6	11	7	61	7	6–61	17.5
Streptococcus bovis 1425	56	53	71	16	46	20	16–71	43.6
Streptococcus pyogenes 1408	8	3	23	26	2	4	2–26	11.0
Staphylococcus aureus 1307	28	70	14	34		4	4–70	30
Staphylococcus citreus 1302	0	0	0	4	10	26	0–26	6.6
Streptococcus faecalis 1435	41	58	111	46	87	23	23–111	61.0
Streptococcus faecalis 1430	24	33	83	41	39	19	19–83	39.8
Streptococcus salivarius St5E	1	0	0	0	0	0	0–1	0.16
Streptococcus salivarius St25D	0	0	2	0	0	0	0–2	0.33

In contrast, *S. faecalis* displayed marked adhesion to the platelets. *S. faecalis*, strain 1435, and *S. faecalis*, strain 1430, caused average platelet count decreases of 79 and 79.5 per cent, and the adhesion indices were 61 and 40, respectively.

S. bovis may also be considered as producing platelet agglutination and adhesion, since the average index of adhesion was 44. In most instances, there was good correlation between the index of adhesion and the platelet decrease, which averaged 81 per cent. With one exception, to be discussed below, *S. mitis* induced considerably less adhesion than did *S. bovis* or *S. faecalis*. The indices of adhesion were 13, 6, 11, 7, 61, and 7, averaging 17.5, as compared to an average platelet count decrease of 41 per cent.

Staphylococcus citreus was included in these experiments in order to obtain data on a nonpathogenic organism. In general, this organism was not very active; however, the indices were variable, being 0, 0, 0, 4, 10, and 26. The average platelet count decrease was 38 per cent, which probably correlates with the adhesion index.

The platelet count decreases and the indices of adhesion of *S. pyogenes* do not appear to be in agreement. This organism caused a definite decrease in the platelet count, averaging 68 per cent, but the indices of adhesion were low, i.e., 2, 3, 4, 8, 23, and 26. Following the rotation of mixtures of blood containing *S. pyogenes*, whitish-gray masses were observed adhering to the sides of the tubes. These masses were teased out with a wire hook and were found to be masses of platelets in which bacteria were enmeshed. In such instances the adhesion index was totally inadequate, and a more accurate indication of the degree of adhesion was obtained from the decreases in the platelet counts, which were 90, 84, 74, 63, 31, and 63 per cent.

The same phenomenon was found to occur when *Staphylococcus aureus* was tested, although masses of mixed platelet-bacteria clumps were not observed in all tubes. The average platelet count decrease was 61 per cent, whereas the adhesion indices were 28, 70, 14, and 4, the average being 30. Because of the errors to which our calculations are subjected, as discussed above, it is not feasible to attempt to decide whether *S. aureus* is more or less adhesive than *S. pyogenes*.

Diplococcus pneumoniae, types VII and I, was studied in four blood samples, the platelets decreasing 37 and 49 per cent, respectively. This platelet count decrease was produced by factors other than the clumping of platelets to bacteria, because mixed platelet-pneumococcus clumping was not seen. It is suspected that soluble substances from pneumococci were responsible for the platelet count decrease.

The results of these tests with rotated blood-bacteria samples show that *S. salivarius* and *D. pneumoniae* are not adhesive to platelets; *S. mitis* and *S. citreus* are moderately adhesive; and *S. bovis, S. pyogenes, S. faecalis*, and *S. aureus* are markedly adhesive.

Rotation of bacteria with isolated platelets. In order to determine whether or not the factor responsible for the adhesion of platelets to bacteria was present in the plasma or in the platelet, bacteria were rotated with platelets which had been washed free of plasma components. These platelets were isolated from citrated rabbit blood by a procedure described previously (Copley and Houlihan, 1946). The platelet count decrease and the index of adhesion were determined for two suspensions of isolated platelets to which had been added *S. salivarius* 1431, *S. mitis, S. bovis, S. pyogenes, S. aureus, S. citreus*, and *S. faecalis* 1435. The results are presented in table 3. Mixed clumping of platelets and bacteria was observed in only 3 of 14 tests, i.e., an adhesion index of 2 was obtained with *S. salivarius* in one of two platelet suspensions, and an index of 4 with *S. bovis* in both platelet suspensions. The platelet count decreases, except in the samples containing *S. salivarius* 1431, were considerably lower than in the whole blood samples containing these organisms. No explanation is apparent for the platelet count decrease of 51 per cent in one platelet suspension containing *S. salivarius* 1431, particularly since very little mixed clumping occurred.

These results indicate that the factor responsible for the agglutination and adhesion of rabbit platelets to bacteria is present in the plasma. This finding verifies the observations of Govaerts (1921*b*) that washed platelets are not adhesive for bacteria.

The effect of bacterial agglutinins on platelet-bacteria adhesion. The agglutinins for each of the bacteria under test were titrated both in the sera of all normal rabbits used and in the sera of rabbits actively immunized with *S. faecalis* 1430, *S. mitis*, and *S. salivarius* 1431. This was done to determine whether there was any correlation between the agglutinins for an organism and its adhesiveness. The agglutinin titers are shown in table 1, and the relationship between platelet count decreases, indices of adhesion, and bacterial agglutinins can be seen in figure 2. The titers expressed as greater than a certain dilution in table 1 have been plotted one dilution higher in figure 2.

The sera of rabbit no. 98, which had been immunized with *S. salivarius* 1431, contained a higher concentration of specific agglutinins than the sera of normal rabbits; nevertheless, the index of adhesion was 0. Neither *S. salivarius* St25D nor *S. salivarius* St5E was found to be very adhesive, yet the agglutinin titers varied, ranging up to 1:256. Although rabbit no. 97 was immunized in a similar manner with *S. mitis*, the agglutinin titer did not increase. Interestingly, the index of adhesion with *S. mitis* in the blood of this rabbit was 61, which was considerably higher than the average index of 8.8 in the other five rabbits. No explanation for this discrepant result can be given.

TABLE 3

Comparison of the adhesiveness of isolated platelets and bacteria with the platelet count decrease in such mixtures

BACTERI	INDEX OF MIXED CLUMPING OF PLATELETS AND BACTERIA		PLATELET COUNT DECREASE IN PER CENT		
	Rabbit no. 1	Rabbit no. 7	Rabbit no 1.	Rabbit no. 7	Average
Streptococcus salivarius 1431	2	0	51	22	36.5
Streptococcus mitis 1432	0	0	2	4	3
Streptococcus bovis 1425	4	4	32	11	21.5
Streptococcus pyogenes 1408	0	0	31		31
Staphylococcus aureus 1308	0	0	23	8	15.5
Staphylococcus citreus 1302	0	0	17	0	8.5
Streptococcus faecalis 1435	0	0	19	22	20.5

Rabbit no. 90 was immunized with *S. faecalis* 1430, which increased the agglutinins from a low level up to a titer of 1:256. The platelet count decrease and the adhesion index in the sera of this rabbit using *S. faecalis* 1430 did not differ significantly from the tests with normal rabbits. The agglutinin titers for *S. citreus* were very low in all rabbit sera, but the adhesion indices ranged from 0 to 26. Probably the most striking example of the lack of correlation between adhesiveness and agglutinin titer is found in the tests in which *S. faecalis* 1435 was used. The indices of adhesion are very high, ranging up to 111, yet, with

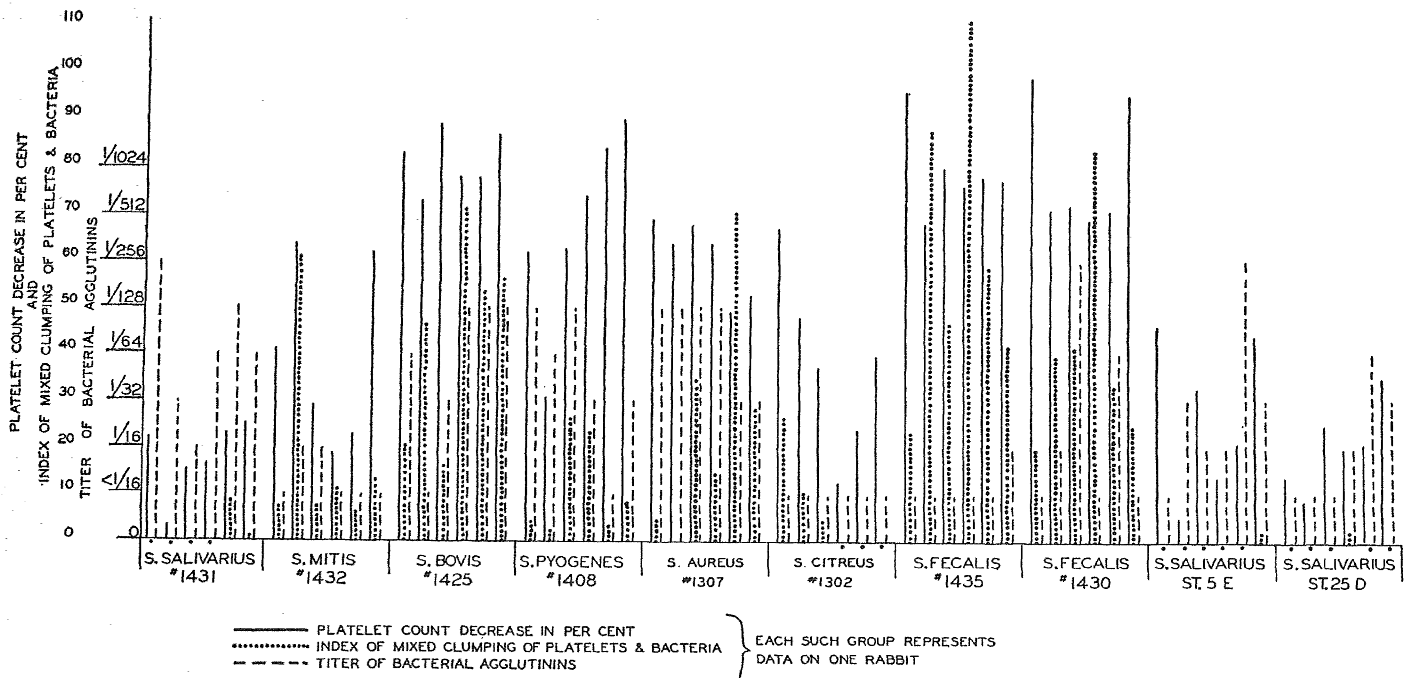

FIG. 2. THE RELATIONSHIP BETWEEN PLATELET COUNT DECREASE, INDEX OF MIXED CLUMPING OF PLATELETS AND BACTERIA, AND TITER OF BACTERIAL AGGLUTININS

one exception, the serum titers are less than 1:16. The relationship in the case of *S. aureus* and *S. pyogenes* is difficult to assess accurately because of the variations in the titers.

DISCUSSION

The results of this study of the adhesion of rabbit platelets and bacteria verify and extend those of previous investigators (Aynaud, 1911; Roskam, 1921; Govaerts, 1921*a*, 1921*b*). The rapidity with which the platelets clumped and enmeshed the bacteria and the firmness of the union (figure 1, nos. 3 and 4), suggest that either very strong chemotactic forces are involved in this reaction or that the bacteria upset the colloidal equilibrium of the plasma. Two points are of interest: (1) evidence of specificity, i.e., all bacteria do not cause the platelets to become unstable and adhesive, and (2) platelets per se are not adhesive, indicating that the factor inducing the clumping and adhesion of platelets to bacteria is present in the plasma. These two facts invite the assumption that we are dealing with a reaction analagous in certain respects to the chemotaxis which exists between specifically sensitized bacteria and leucocytes. However, for several reasons, discussed below, it is doubtful whether the mechanisms responsible for phagocytosis and for adhesion of platelets to bacteria are comparable in any respect other than that mentioned. Current investigation has revealed that the degrée of adhesion of dog platelets to bacteria varies in citrated, but not in oxalated, blood. It is not known whether this same phenomenon occurs in rabbit blood. Following this thought, Roskam (1927) has shown that sodium citrate in high concentrations does depress the adhesion reaction. We verified the report of Delrez and Govaerts (1918) that pneumococci which had been sensitized with specific agglutinins did not adhere to rabbit platelets. In addition, we tested bacteria which were adhesive in an attempt to determine whether specific agglutinins might enhance the reaction. The results indicate that the adhesive factor is not related to, or dependent upon, the action of bacterial agglutinins. Govaerts' (1921*b*) observation that the plasmatic factor inducing adhesion is heat-labile offers additional evidence of this contention.

As stated, the reason for the variations in the intensity of the adhesion reaction displayed by different strains is not known. Although bacteria possessing varying degrees of virulence were used, no relationship could be established between virulence and adhesion. In addition, it would seem that soluble bacterial products are not responsible for the adhesion phenomenon, since sedimented, washed, formalized cultures were used throughout these tests. With few exceptions, this assumption was substantiated by the correlation of platelet count decreases with platelet-bacteria adhesion indices. Certain physiologic properties of these organisms do not appear to be correlated with the adhesiveness of the organisms. For example, *S. faecalis* and *S. aureus* are more resistant to heat and chemicals, such as penicillin, than *S. pyogenes* (Rorimer, Houlihan, and Lawson, 1945); yet each of these three species is very adhesive.

In another report (Houlihan and Copley, 1946) we have shown that rabbit platelets are stable in rotated citrated rabbit plasma, but unstable in heparinized blood. The fact that rabbit platelets are stable in citrated blood, yet clump and adhere to bacteria introduced into such blood, suggests that different plasmatic factors may be involved in the agglutination of platelets, which may take place in the absence of foreign particulate matter, and the adhesion of platelets to bacteria. Investigation is being conducted currently in order to acquire additional information on this point.

The problem of producing experimental bacterial endocarditis has occupied many investigators for some time. Usually bacterial strains, recently isolated from human cases, have been inoculated intravenously into normal or treated rabbits or dogs. However, there is a paucity of data concerning the mechanism responsible for the adhesion of the cellular elements of the blood, particularly platelets, to the bacteria. Further study of these adhesion reactions may aid future approaches to experimental endocarditis in animals, and also may advance our present concepts of the pathogenesis of bacterial endocarditis in man, or perhaps of any disease in which there is bacterial invasion.

SUMMARY

When certain bacteria are mixed and rotated with whole citrated rabbit blood, the platelets rapidly and intensely clump and adhere to them. *Streptococcus salivarius* induces very little or no platelet agglutination or adhesion; *Streptococcus mitis* and *Staphylococcus citreus* are moderately active in producing this phenomenon; and *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus pyogenes*, and *Staphylococcus aureus* are very active.

Platelets which have been isolated from the blood and washed free of plasma do not adhere to bacteria.

Bacterial agglutinins, present in the sera of normal or actively immunized rabbits, are not responsible for the mixed clumping of platelets and bacteria.

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CETYL PYRIDINIUM CHLORIDE

II. AN IN VIVO METHOD OF EVALUATION

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Evaluation of the germicidal efficiency of the quaternary ammonium compounds by the method given in Circular no. 198 of the U. S. Department of Agriculture is somewhat difficult. Precise, consistent results cannot be obtained, and an "average" of several tests must usually be taken before a final result can be determined. In this paper an *in vivo* method for the evaluation of the quaternary ammonium germicides is described.

EXPERIMENTAL

Cetyl pyridinium chloride was used for the test compound. Killing dilutions of the compound were determined for a virulent strain of *Salmonella typhimurium* by the *in vitro* method described in Circular no. 198 of the U. S. Department of Agriculture. The results of these tests were compared with results obtained by injecting intraperitoneally, into white mice, bacteria which had been treated with the various germicide dilutions for 5 and 10 minutes. The latter test is essentially a duplication of the former test except that "subcultures" were made by inoculating white mice instead of FDA broth. Each mouse was injected intraperitoneally with 0.25 ml of germicide-bacteria mixture plus 0.25 ml of 3 per cent sterile mucin. Five hours after inoculation the mice were anesthetized, and specimens of heart blood and peritoneal fluid were obtained aseptically and cultured in FDA broth. On the following day positive broth cultures were streaked on bacto SS agar plates and bismuth sulfite agar. The presence of organisms of the genus *Salmonella* was confirmed with Kligler's iron agar slants. Recovery of the viable pathogen from the experimental animal indicated lack of germicidal action, whereas failure to recover the organism indicated bactericidal effect in the corresponding germicide dilution. Ten tests were performed for the *in vitro* studies (table 1). A killing dilution of 1:50,000 was obtained in four tests. The other tests show the type of erratic results frequently observed when the FDA procedure is employed for the evaluation of the germicidal effectiveness of these compounds.

Ten tests were performed by the *in vivo* technique (table 1). By this procedure killing dilutions ranged from 1:30,000 to 1:60,000. The average killing dilution was 1:45,000. This result compares favorably with the killing dilution as determined *in vitro*.

TABLE 1

Killing dilutions of cetyl pyridinium chloride at 37 C for Salmonella typhimurium

TEST NO.	IN VITRO (FDA METHOD)	IN VIVO (INJECTED INTO 20- TO 25-MG WHITE MICE)
1	1:90,000	1:35,000
2	no end point	1:45,000
3	<1:50,000	1:60,000
4	<1:50,000	1:60,000
5	1:50,000	1:30,000
6	1:50,000	1:50,000
7	1:50,000	1:50,000
8	1:50,000	1:50,000
9	no end point	1:40,000
10	no end point	1:30,000
Average..................	1:58,000	1:45,000

DISCUSSION

The erratic results frequently obtained with *in vitro* methods emphasize the need for comparative tests for the evaluation of the germicidal potency of quaternary ammonium salts. The *in vivo* method described above provides a useful comparative test as well as information about cationic germicides under conditions similar to those found in medical practice. In spite of the shortcomings of the FDA technique, the *in vitro* results are corroborated by the *in vivo* results. Although killing dilutions as determined by the *in vivo* test are not identical from day to day, any given test shows a sharp end point and there are no "wild plusses" or unreadable results.

Valko and Dubois (1944) have shown that, within narrow time limits, bacteria treated with quaternary ammonium salts may be detoxified by anionic detergents, and that lethal action of the germicides may thus be prevented. This type of inactivation may be accomplished only by specific laboratory techniques and under circumstances which do not exist under conditions of ordinary usage. If killing dilutions as determined by the *in vitro* test were consistently higher than killing dilutions as determined by the *in vivo* test, detoxification of the germicide-treated organisms by body fluids would be indicated. Since, however, the results by both tests were comparable, no reversal of germicidal action by body fluids could be postulated. Bacteria treated with killing dilutions of cetyl pyridinium chloride are not "revived" by contact with body fluids and tissues. As judged by the *in vivo* test, the action of this quaternary germicide is bactericidal rather than bacteriostatic.

SUMMARY

An *in vivo* method for the evaluation of the germicidal potency of quaternary ammonium salts is described.

For the test organism, *Salmonella typhimurium*, the results obtained *in vivo* correlate well with the results obtained by the FDA test.

The test organism after treatment with cetyl pyridinium chloride was not detoxified by contact with body fluids and tissues.

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ON THE ISOLATION FROM AGAR OF AN INHIBITOR FOR NEISSERIA GONORRHOEAE

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The separation of an inhibitor for *Neisseria gonorrhoeae* from commercial agars was attempted on the basis of previous work by Mueller and Hinton (1941) and Gould, Kane, and Mueller (1944). In addition, this inhibitor was compared with certain organic compounds of a similar nature by biologic tests of inhibitory activity.

Until the medium of Mueller and Hinton (1941) was developed, it was thought impossible to grow *N. gonorrhoeae* on a simple, well-defined medium, but these investigators showed that casein hydrolyzate, meat extract, agar, and starch supported growth of the organisms as well as chocolate blood agar or ascitic fluid agar. Gould, Kane, and Mueller (1944) further showed that a fluid medium containing casein hydrolyzate, sodium chloride, magnesium sulfate, and phosphate buffer would support growth of *N. gonorrhoeae*, and that a similar medium with agar added would fail to grow the organisms. The addition of starch to this last medium again permitted growth, which led the authors to the hypothesis that starch neutralized an inhibitor present in the agar. Frantz (1942) also developed a similar simple fluid medium for a closely related organism, *Neisseria intracellularis*, and observed inhibition of growth of the organisms upon addition of agar to the medium. The experimental work which follows was begun in order to prove the hypothesis of inhibition of *N. gonorrhoeae* by agar, or a substance in it, that was proposed by Gould, Kane, and Mueller.

EXPERIMENTAL

Separation of the inhibitor from agar. In the following preparations Merck's U.S.P. agar-agar shreds were used throughout. It was decided to attempt the separation of the inhibitor from the agar by the customary methods of dialysis and extraction. Hydrated agar was dialyzed through viscose tubing against running tap water for 3 days, and a similar sample of agar was electrodialyzed, using viscose partitions, for a similar length of time. Solid hydrated agar, cut in 1.0-cm cubes, was extracted for 7 days by direct contact with large volumes of distilled water, 0.1N HCl, 0.1N NaOH, and 95 per cent ethanol. A series of 5.0-g lots of dry, shredded agar was extracted for 3 days each in an all-glass Soxhlet apparatus, having an extraction chamber of 100-ml capacity and 40-mm diameter, with 250 ml of each of the following freshly distilled cp solvents: diethyl ether, acetone, benzene, chloroform, ethanol, and methanol.

The treated agar samples were incorporated into the medium of Mueller and Hinton (1941) without starch in the following proportions:

Agar	2.5 g
Distilled water to make	46 ml

Neutralize this mixture to litmus with 1 N NaOH or HCl and autoclave 15 minutes at 15 pounds' pressure to put the agar into solution. (In hydrated agar the amount of water present may be determined by weighing, and a suitable amount added to bring the total volume to 46 ml.)

Casein hydrolyzate, Difco	2.58 g
Meat infusion[1]	44 ml

Add this mixture to the warm fluid agar, bring to pH 7.4 by the addition of 1 N NaOH, tube in 13.5-ml amounts, and autoclave 10 minutes at 10 pounds' pressure.

When the medium was to be used, the tubes were heated in boiling water for 5 minutes to melt the agar; 1.5 ml of 0.9 per cent sterile saline or 1.5 per cent starch sol[2] were added as required; and the contents of the tubes were poured into sterile petri dishes, which were rotated to mix the addition and the agar. After the medium had hardened, it was inoculated in sectors with one loop of a heavy saline suspension of a 24-hour growth of the test strain of gonococcus from a starch agar slant.[3] The sector was streaked with a loop in the customary manner. The plates were incubated in a candle jar at 35 C for 24 hours, and then were examined.

Through experience it has been found that colony size is a poor criterion of the efficacy of the medium or, to put it another way, of the presence of inhibition. The pattern of growth furnishes a much better criterion. The organism may not have grown at all; it may have grown only at the start of the streak in the region of large inoculum; or it may have grown along the entire streak in the area of small as well as of large inoculum. These three responses are indicated, respectively, by the letters O, L, and S in tables 1 and 2 below. In general, it may be said that where there is no inhibition the growth is S; where there is moderate inhibition, L; and where there is much inhibition, O.

Table 1 demonstrates that neither dialysis nor electrodialysis is effective in removing the hypothetical inhibiting agent from agar. Extraction by contact with water, acid, and alkali, as well as by ethanol, is similarly ineffective. In all cases the organisms grew well on the treated agar with added starch, indicating that the procedures did not introduce further inhibiting substances not neutralized by starch.

Table 2 demonstrates that with the exception of diethyl ether, the organic solvents did not introduce additional inhibitory substances, for growth was excellent on the media containing the added starch. Without the starch, however, growth was poor in all agars extracted with organic solvents except methanol. In that one instance growth was almost equivalent to that on the same medium with added starch. It may be concluded that methanol extraction removes the inhibiting agent for *N. gonorrhoeae* that is present in commercial agar.

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[1] Meat infusion may be prepared by suspending 1 pound of chopped beef heart in 500 ml of tap water, which is brought to active boiling, and then filtered through cheesecloth and filter paper.

[2] Starch sol may be prepared by making a paste of 1.5 g of corn starch (Argo) in 10 ml cool, distilled water, and pouring the paste with stirring into 90 ml boiling distilled water. Autoclave 10 minutes at 10 pounds' pressure.

[3] Through the course of this experiment 19 strains of *N. gonorrhoeae* were used, all supplied by the Boston Dispensary. All were checked by colonial and microscopic morphology and fermentation reactions.

TABLE 1

The effect of dialysis and simple extraction of agar on the growth of gonococci

PREPARATION OF AGAR	GONOCOCCUS STRAINS					
	3	5	7	3	5	7
	Starch added			Saline added		
None	S	S	S	L	L	L
Dialysis, tap water	S	S	S	L	0	0
Electrodialysis	S	S	S	0	0	0
Extraction, H_2O	S	S	S	0	S	L
Extraction, HCl	S	S	S	L	L	L
Extraction, NaOH	S	S	S	0	L	L
Extraction, EtOH	S	S	S	0	S	L

TABLE 2

The effect of extracting agar with organic solvents on the growth of gonococci

EXTRACTING AGENT*	GONOCOCCUS STRAINS								
	11	12	13	14	15	16	17	18	19
	Starch added								
None	S	S	S	S	S	S	S	S	S
Ether	S	S	S	S	0	0	0	S	0
Acetone	S	S	S	S	S	S	S	S	S
Benzene	S	S	S	S	S	S	S	S	S
Ethanol	S	S	S	S	S	S	S	S	S
Methanol	S	S	S	S	S	S	S	S	S
	Saline added								
None	0	0	S	S	0	S	0	S	0
Ether	0	0	S	L	0	S	0	L	0
Acetone	0	0	L	L	0	S	0	L	L
Benzene	L	0	S	S	L	S	0	S	0
Ethanol	0	0	0	0	0	0	0	0	L
Methanol	S	S	S	S	S	S	S	S	0

* The chloroform-extracted agar was omitted from the experiment because it did not solidify, presumably because of acid hydrolysis by the decomposing solvent.

At this point in the investigation it was decided that the easiest method of quantifying the inhibitory activity of a methanol extract of agar would be to use a suitable fluid medium which would reproducibly grow the organisms, and to which could be added decimal dilutions of the extract. In this manner the minimum amount of extracted material inhibiting growth could be determined. The medium of Mueller and Hinton (1941) seemed an excellent one to modify; the agar and starch were omitted from it, giving it the following composition:

Distilled water	300	ml
Meat infusion[4]	150	ml
Casein hydrolyzate, Difco	8.75	g

Bring this mixture to pH 7.4 with 1 N NaOH, and, after placing in suitable containers, autoclave for 10 minutes at 10 pounds' pressure.

Ten ml of the medium were first placed in 20-mm test tubes, but inconsistent results in terms of reliable growth following a 0.5-ml inoculum of a 24-hour fluid culture directed attention to other forms of containers. A 10-ml volume of fluid in a 50-ml Erlenmeyer flask was then tried, with consistent growth following a 0.5-ml inoculum. These flasks were carefully cleaned in a hot conc. $H_2SO_4 = HNO_3$ bath and rinsed thoroughly with distilled water each time before use. They, like the plates previously described, were incubated for 24 hours at 35 C in a candle jar.

When the organisms did grow in the test tubes, they were viable at 35 C for as long as 8 days, whereas those in the flasks were viable only 3 days. The main difference in the two cases was in the surface-volume ratio for the medium, so the phenomena observed may have been due to a difference in gas exchange at the gas-liquid interface. This hypothesis has not yet been investigated. It may be pointed out, however, that Frantz (1942) encountered a similar difficulty in cultivating the meningococcus in fluid media. In the flasks, the Mueller-Hinton fluid modification was a quite satisfactory and useful tool with which further work on extraction could be pursued.

Characterization and estimation of activity of inhibitor. Forty g of Merck's shredded agar were extracted in a Soxhlet apparatus with cp methanol for three days, and the volume of the extract was reduced to 80 ml by distillation. This extract has a light brown color and no recognizable odor. When 10 ml of the extract were added to an equal volume of water, a curdy, white precipitate formed, which dissolved upon heating and the addition of NaOH, and which reappeared upon acidifying with H_3PO_4. Upon extraction of the acid mixture with petroleum ether, the precipitate disappeared from the aqueous phase. This information indicated that the methanol extract contained an appreciable concentration of a fatty-acid-like compound.

Further extraction of the compound from acid and alkaline aqueous phases by petroleum ether was unsatisfactory because of highly persistent and troublesome emulsions of the two phases. However, the biologic activity of the product of the methanol extraction was tested by evaporating a volume containing the extract of 2.5 g of agar to dryness on the steam bath, adding 2.0 ml of water to the residue, and neutralizing the solution to phenol red indicator with NaOH and H_3PO_4. The solution was then evaporated to one-half its volume over an open flame, a procedure which effectively sterilized it. A series of flasks containing 9.0 ml of fluid medium was set up as noted in the protocol below, and after inoculation with 0.5 ml of a 12-hour fluid culture of gonococcus strain 11, which was known to be sensitive to agar inhibition, the flasks were incubated for 24 hours in a candle jar at 35 C. This was followed by streaking a loop from each of the flasks on a sector of a Mueller-Hinton starch agar plate to demonstrate the growth of the inoculum. The results following incubation of the plates at 35 C for 24 hours in a candle jar are shown in table 3.

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[4] See footnote 1.

From these data it may be concluded that a methanol extract of agar contains a substance which, in suitable concentrations, inhibits completely the growth of gonococcus strain 11 and which is biologically neutralized by the addition of starch.

After removing an aliquot containing 1.0 mg solids, the methanol extract of 30 g of agar containing 90 mg of solid material was made alkaline with NaOH and reduced to dryness on a steam bath. The residue was treated as indicated in the following flow sheet.

TABLE 3

The effect of products of methanol extraction of agar on the growth of gonococcus strain 11 in fluid medium

ADDITION	INOCULATED	STARCH SOL 1.5% 4.5 ML	PLATE GROWTH
None	−	−	0
None	−	+	0
None	+	−	++++
None	+	+	++++
Methanol extract 1.25 g agar	+	−	0
Methanol extract 1.25 g agar	+	+	++++

90 mg solid material

Heat 98 C for 4 hr
to char polysaccharides

Extract 3x with 1.5 ml
petroleum ether —— Extract: discard

Residue: acidify with
2.0 ml 0.1 N HCl

Extract 3x with 2.0 ml
petroleum ether —— Residue: discard

Extract: heat to dryness
on steam bath, yield 3.0 mg
of white solid

The 3.0 mg of white solid was dissolved in 50 per cent aqueous ethanol and titrated with 4×10^{-2} N NaOH, using phenolphthalein as an indicator. The calculated equivalent molecular weight, based on the assumption of one carboxylic group per molecule, was 3.7×10^2. This would suggest that the compound contains 24 carbon atoms per molecule, if it is a straight chain, aliphatic compound.

Because it was felt that the inhibiting compound obtained from agar was similar to a fatty acid, a crude mixture of butter fatty acids was prepared by alkaline hydrolysis of domestic butter. In addition, cp stearic acid (Eastman) and oleic acid (Merck) were procured for comparison with the inhibitor. These acids and the inhibitor were put into solution in suitable concentrations in 2.0 ml of water adjusted to approximate pH 7.4 with phenol red indicator by the addition of 0.5 N Na_2CO_3 and 1 M H_3PO_4. The solutions were reduced to one-half their volume by boiling, which effectively sterilized them, and were added with bacteriologic technique to a series of 50-ml Erlenmeyer flasks containing 9.0 ml of fluid medium. Each of the fatty acids was serially diluted in the same quantity of medium so that a wide range of final concentrations of the fatty acids was obtained. All the flasks were inoculated with 0.5 ml of a 12-hour fluid culture of gonococcus strain 11, and were incubated 12 hours at 35 C in a candle jar. One loop of fluid from each flask was then removed and streaked on a starch agar plate to test for growth of the inoculum. These plates were examined, following 24 hours of incubation at 35 C in a candle jar (table 4).

TABLE 4

Relative growth of gonococcus strain 11 in the presence of various concentrations of fatty acids

MATERIAL ADDED	CONCENTRATION OF ADDITION (Dry wt, µg/ml)							
	100	50	10	5.0	1.0	0.5	0.1	0.05
1 mg aliquot of orig. extract		0		++++		++++		
Purified white solid		0		0		0		++++
Butter fatty acids	0		0		0		++	
Oleic acid	0		0		0		++++	
Stearic acid	0		0		+		++++	

On the basis of these data, it may be concluded that the procedure of purification of the methanol extract concentrated the inhibiting fraction approximately 100 times. If the number of units of inhibition in the original extract and in the purified product is calculated (defining a unit as the minimum amount inhibiting growth under the conditions of the experiment), it is found that the original extract contained 1.8×10^3 units, and the product, 6.0×10^3. For the crude biologic assay employed, the agreement is excellent, but even more important is the implication that very little active material was lost in purification. Oleic, stearic, and butter fatty acids may be seen to have an inhibitory action similar to that of the purified agar extract in essentially the same concentrations. It has been shown, in addition, that the activity of both the fatty acids tested and the agar inhibitor is fully retained following refluxing for 1½ hours with 2.5 N NaOH.

The role of starch. The work of Mueller and Hinton (1941) did not explain the protective action of starch in their medium, but Schoch and Williams (1944) have demonstrated what appears to be the probable mechanism of action. These authors described the adsorption of fatty acids by the linear component of corn starch; the adsorption was reversible, and by the use of either 80 per cent aqueous dioxane or methanol the acids could be recovered quantitatively from the starch. The Corn Products Refining Company kindly supplied samples of various commercially available starch products, which were tested for activity in the Mueller-Hinton medium. It was found that mildly hydrolyzed derivatives and the linear component of whole starch were as effective as the unmodified product. All strongly hydrolyzed starches and glycogen were ineffective in the medium. All effective products gave a blue color when tested with iodine, but one starch which was not satisfactory for the medium also gave a blue color. This confirms the impression of Mueller and Hinton (1941) that commercial starch lost its activity when hydrolyzed by ptyalin to the point at which it no longer gave a blue color with iodine.

SUMMARY AND DISCUSSION OF RESULTS

The development of the starch agar medium of Mueller and Hinton (1941) and the further clarification of the growth requirements of the gonococcus by Gould, Kane, and Mueller (1944) seemed to indicate that commercial agar contained an inhibitory compound that rendered growth of the gonococcus difficult without starch. Not all strains of gonococcus were sensitive to the inhibitor, and, indeed, of the 19 strains used in the work reported in this paper, one-half were not sensitive to the inhibitor in its usual concentration in 1.75 per cent agar media. Separation of this inhibitor from the agar was successfully accomplished by continuous methanol extraction, and the compound was found to have many of the properties of fatty acids. It was further concentrated by extraction with petroleum ether to the point that 0.5 μg per ml was sufficient to inhibit growth completely. Oleic, stearic, and butter fatty acids were shown to have similar inhibitory properties in equivalent concentrations. The actual chemical composition of this inhibitor has not been determined, but its solubility properties, its approximate equivalent molecular weight, and its biologic similarity to two of the higher fatty acids would indicate that it resembles these fatty acids very closely.

The mechanism of neutralization of the inhibition by starch is postulated to be an adsorption phenomenon on the basis of the work of Schoch and Williams (1944). Glass and Kennet (1939) have shown in addition that certain varieties of blood and sugar charcoals, as well as graphite, permitted growth of gonococci in the media which they described. Charcoals have long been known to be excellent adsorbers of fatty acids, and Linner and Gortner (1935) employed "norite" to check the Langmuir adsorption equation for 31 organic compounds, including 6 fatty acids. The actual mechanism of inhibition of growth of the gonococcus by fatty acids is unknown at present. Several authors, Dubos (1945), Dubos and Davis (1946), and Wyss, Ludwig, and Joiner (1945), have described the inhibiting effects of fatty acids on other microorganisms, so that it is possible that other organisms may be found equally susceptible to this inhibiting agent in agar. This does not mean that the fatty acid has possibilities as a new chemotherapeutic agent; the varying susceptibility of the gonococcus and the ease with which the material may be neutralized suggest that its use in clinical medicine would be extremely limited. It is felt, too, that extraction of all bacteriologic agar to remove the inhibiting acids is not justified in view of the favorable results with the addition of starch to a medium like the Mueller-Hinton.

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UREA DECOMPOSITION AS A MEANS OF DIFFERENTIATING PROTEUS AND PARACOLON CULTURES FROM EACH OTHER AND FROM SALMONELLA AND SHIGELLA TYPES[1]

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Bacterial decomposition of urea has been especially useful in distinguishing between members of the *Proteus* group and other fecal organisms encountered in stool examinations. Originally, the reactions of these organisms on urea were determined by the use of sterile urine as a culture medium, and only recently have media of known composition been employed (Rustigian and Stuart, 1941). Of the media of known composition, those of Rustigian and Stuart and of Ferguson and Hook (1943) have been found suitable to differentiate between *Proteus* and other organisms believed to be urease-negative.

In considering the formulae of the two media mentioned, it is obvious that the medium of Ferguson and Hook (urea, 2 per cent; phosphate buffer; NaCl, 0.5 per cent; and ethyl alcohol, 1.0 per cent) is of such a nature that only an organism capable of utilizing urea as a sole source of nitrogen would grow. The possibility therefore arises that this medium would not detect organisms which could hydrolyze urea but which could not utilize the liberated ammonia as a source of nitrogen. In such a case, no growth would occur, and the organism would be considered urease-negative.

The medium of Rustigian and Stuart (urea, 2 per cent; yeast extract, 0.01 per cent; and phosphate buffer), because of the extremely small amount of nutritive material present, also is subject to the same theoretical limitations, particularly in view of the fact that an organism incapable of utilizing ammonia is probably forced to use the yeast extract not only as a nitrogen source but also as a carbon source. If these materials are exhausted before the organism shows appreciable growth, its ability to hydrolyze urea cannot be adequately determined. The high buffer capacity of the medium would also mask slight urease activity if such an organism were capable of initial growth in the medium.

These two media, however, as stated by the authors, were designed for the detection of *Proteus* organisms, and for this purpose they are well suited. It was thought possible, however, that if a medium were devised which eliminated the necessity of an organism's utilizing ammonia as a sole source of nitrogen, other organisms of the fecal group could be detected which would hydrolyze urea.

MEDIUM AND METHODS OF TESTING

The medium devised consists of urea, peptone, sodium chloride, monopotassium phosphate, glucose, phenol red, and agar in proper proportions in distilled water. Urea hydrolysis in this medium is shown by a change in color from the pale yellow of the fresh medium to an intense red-violet color.

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[1] This investigation was made in the Laboratory Service of the 6th General Hospital, Casablanca, North Africa.

[2] Captain, U.S.A. Reserve, Retired.

Preliminary experiments showed that if the quantities of peptone ordinarily used in media were employed (0.5 per cent), the false reactions described by Ferguson and Hook in Ashworth's medium were encountered. However, it was thought that by decreasing the quantity of peptone used, and by adding glucose, the false reactions might be eliminated. The acidity from glucose fermentation would thus counteract the alkalinity produced by peptone decomposition, and the glucose would in addition furnish a readily available source of energy, further protecting the peptone from decomposition. To test this medium for false positive reactions, and to determine whether or not the acid produced from glucose would lead to false negative results, three controls were used, together with the final medium, as follows:

I. Control (Base)

Peptone, bacto	1 g
Sodium chloride	5 g
Monopotassium phosphate	2 g
Phenol red	0.012 g
Agar	20 g
Distilled water	1,000 ml

II. Control
Base + 0.1 per cent glucose
III. Control
Base + 2 per cent urea
IV. Final medium
Base + 0.1 per cent glucose + 2 per cent urea

The base medium (I) and the base medium with glucose (II) were adjusted to pH 6.8 to 6.9, tubed, and sterilized at 15 pounds' pressure for 20 minutes. A 20 per cent solution of urea (Mallinckrodt, analytical reagent) was sterilized by passage through a Seitz filter, and enough was added to tubes of I and II, after they were cooled to approximately 50 C, to make up III and IV with a final concentration of 2 per cent urea.

A rough estimation of the rate of urease activity in *Proteus* and other coliform organisms was made in the following manner: Media were tubed in approximately 5-ml quantities, and the tubes were slanted so as to leave a butt of about 1 inch in depth with a slant of about 1.5 inches in length. The media were inoculated heavily by spreading a bit of growth from a positive Kligler agar culture over the entire slant. Organisms which hydrolyzed urea produced the characteristic red-violet color on the slant, and, as incubation proceeded, the color extended toward the bottom of the tube. The extent of color penetration into the medium was taken as a measure of urease activity and was recorded as plus and minus signs, as follows: If the color had developed just beneath the surface of the slant but had not penetrated further into the medium, the reaction was designated + − −. If it had penetrated to the junction of the bottom of the slant with the butt, it was designated +. Further penetration into the butt was designated ++, +++, and ++++, the latter symbol being used when the color had reached the bottom of the tube.

ORGANISMS USED AND RESULTS

In addition to the various *Proteus* species, members of the paracolon, *Salmonella*, and *Shigella* groups were tested on the media. The paracolon cultures were classified as paracolon *Aerobacter*, paracolon intermediates, or paracolon *Escherichia* on the basis of biochemical characteristics. All the cultures tested were freshly isolated from stool specimens in connection with another investigation.

TABLE 1

Cultural reactions of test organisms on control and urea media

ORGANISMS	NO. OF STRAINS TESTED	BASE = I			BASE + GLUCOSE = II			BASE + UREA = III			BASE + GLUCOSE + UREA = IV		
		6 hr	1 day	6 days	6 hr	1 day	6 days	6 hr	1 day	6 days	6 hr	1 day	6 days
Proteus vulgaris	7	N	N	YO	Y	Y	Y-N	R++	R ++++		R +++	R ++++	
Proteus mirabilis	8	N	N	YO	Y	Y	N-YO	R++	R ++++		R +++	R ++++	
Proteus morganii	5	N	N	YO	Y	Y	Y	R++	R ++++		R ++	R ++++	
Proteus rettgeri	4	N	N	YO	Y	Y	Y	R++	R ++++		R +++	R ++++	
Paracolon *Aerobacter*	10	N	N	YO	Y	Y	Y-N	N	N	YO-OP	Y-R +−	Y-R+	R+ to R ++++ 3-5
Paracolon intermediate	11	N	N	YO-O	Y	Y	Y-N	N	N	O-OP	Y−R +−	Y−R+	R+ to R++++ 3-5
Paracolon *Escherichia*	23	N	N	YO-O	Y	Y	N-YO	N	N	YO-OP	Y	Y	Y-YO
Salmonella species	2	N	N	YO	Y	Y	N	N	N	YO	Y	Y	N
Shigella species	9	N	N	N-YO	Y	Y	Y-N	N	N	N-YO	Y	Y	Y

N = color of uninoculated control. Y = yellow, YO = yellow-orange, O = orange, OP = orange-pink, and R = red-violet Plus and minus signs indicate extent of penetration of color into medium (see text). Letters with hyphens between indicate variations of different strains. Numbers in last column indicate number of days for 4 + reactions to develop.

Cultural reactions of the test organisms on the urea medium and the controls are shown in table 1. All media were inoculated as described and incubated at 37 C. Observations were recorded at the end of 6 and 24 hours, and every day thereafter for 6 days. For the sake of brevity, only the 6- and 24-hour and the 6-day periods of incubation are given in the table. Reactions are recorded as letters which represent the colors of the media at the incubation periods shown. Tubes of uninoculated media were incubated at the same time. Inoculated controls were observed to go through all or part of the following color changes: Y ⇄ N → YO → O → OP. The meaning of these letters is given under table 1. Uninoculated media showed no appreciable color change after 6 days' incubation.

The letter R is used to represent the red-violet color produced by definite urea hydrolysis. The difference between this color and the deepest color (OP) produced on the control media is striking. Furthermore, in only one culture of the 45 urease-positive cultures tested was this color developed gradually over the 6-day period. In all other cultures it was produced abruptly (overnight) from a negative slant, then it penetrated either rapidly or slowly into the body of the medium, depending on the rate of urea hydrolysis.

The data in table 1 show that the moderate alkaline reaction produced by the test organisms on the basal medium (I), even though not of sufficient intensity to be confusing, is effectively counteracted by the addition of glucose (medium II). On the other hand, the presence of glucose not only fails to give false negative reactions through acid production, but actually stimulates urease activity in the slow urea-splitting paracolon organisms (medium IV). In no case was the characteristic red-violet color developed by the latter organisms on medium III, though there was a greater color development after 6 days (OP) than was shown by urease-negative organisms. Hence, glucose apparently increases the sensitivity of the medium in detecting weak urea-splitting organisms. The stimulating action is probably due to the increased rate of metabolism and cell reproduction accompanying such a readily available source of energy. The fact that the stimulating effect is slight in the *Proteus* group suggests that the urease in that group is perhaps a strong constitutive enzyme of considerable importance in the normal metabolism of the cell.

The new medium demonstrates definite urease activity in the paracolon *Aerobacter* and paracolon intermediate organisms tested. This is not surprising in view of the fact that normal *Aerobacter* and intermediate organisms are known to be able to utilize urea as a sole source of nitrogen (Vaughn and Levine, 1942). It might be expected that though these organisms in their evolutionary development might lose their capacity to utilize urea ammonia as a sole source of nitrogen, they could retain or quickly regain their ability to hydrolyze urea.

There is a marked difference between *Proteus* and the urease-positive paracolon organisms in the rate of urea hydrolysis as determined by this medium. The majority of *Proteus* organisms within 1 hour after inoculation gave R+ − − reactions, and within 6 hours the color had penetrated deeply into the medium. All *Proteus* species gave R+ + + + reactions after 24 hours. Members of the urease-positive paracolon organisms varied from a few which gave only a R+ reaction after 6 days, indicating delayed ability to hydrolyze the urea, to those which gave R+ − − reactions in 6 hours, developing slowly to R+ + + + reactions after 3 to 5 days.

Within the genus *Proteus* itself no marked difference between the various species in rates of urea hydrolysis was detected. Rustigian and Stuart (1941) have reported that *Proteus morganii* was distinctly slower in hydrolyzing urea than were other species of the group. Although comparatively few strains of this species were tested in the present study, it appears that, under the more suitable conditions of growth offered by the new medium, the organism hydrolyzes urea as readily as do other members of the group.

The paracolon *Escherichia* cultures tested did not hydrolyze urea. It would not be surprising, however, in view of the cultural variations among paracolon organisms, to find occasional paracolon *Escherichia* cultures capable of hydrolyzing urea, and conversely to find occasional paracolon *Aerobacter* and paracolon intermediate cultures which fail to hydrolyze the compound. The data presented here, however, indicate that most paracolon *Aerobacter* and paracolon intermediate cultures are urease-positive, and that paracolon *Escherichia* cultures are urease-negative.

The *Salmonella* and *Shigella* species tested in this investigation and in subsequent routine use of the medium over a period of 6 months were never found to produce even a trace reaction on the medium described. Ewing (1946) subsequently used the medium routinely in the examination of thousands of feces specimens and confirmed these findings.

It is believed that the medium described is of distinct value as a routine aid in enteric bacteriology. Its usefulness lies in its convenience, dispensing as it does with further manipulation after it has been inoculated, and in its ability to demonstrate hydrolysis of urea in *Proteus* organisms within 1 to 6 hours and in paracolon *Aerobacter* and paracolon intermediate organisms usually within 24 hours. These organisms can thus be eliminated from subsequent routine serological investigations without further time- and material-consuming tests.

The medium, however, cannot be used to determine absolute rates of urease activity, nor, it is believed, can any medium thus far described. The fact that most of the urease-positive paracolon organisms tested in this study hydrolyzed urea within 24 hours, and then proceeded slowly to decompose the urea further, as shown by the slow penetration of the alkaline color of the indicator into the medium, may be merely an indication of lack of tolerance of the organisms to increased alkalinity, with subsequent depression of urease activity. Final word on absolute rates must therefore await further investigation.

SUMMARY

A urea medium is described which eliminates the necessity of an organism's utilizing urea ammonia as a sole source of nitrogen, and which is therefore capable of demonstrating urease activity in organisms somewhat more fastidious in their nitrogen requirements. The medium shows that, in addition to *Proteus*, paracolon *Aerobacter* and paracolon intermediates are definitely urease-positive. Paracolon *Escherichia* as well as *Salmonella* and *Shigella* species are urease-negative.

The medium is recommended as an aid in routine enteric bacteriology because

of the convenience of its use, the rapidity with which it detects paracolon *Aerobacter* and paracolon intermediate organisms, as well as *Proteus*, making it possible to eliminate these cultures from further serological tests. Theoretical aspects of urea decomposition as shown by this medium are discussed.

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THE INFLUENCE OF THIAMINE ON THE SUSCEPTIBILITY OF CHICKS TO AVIAN ENCEPHALOMYELITIS[1]

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Previous reports from our laboratories have dealt with the relation of nutrition to the resistance of rhesus monkeys and of Swiss mice to virus infections, poliomyelitis (Rasmussen, Waisman, Elvehjem, and Clark, 1944; Lichstein *et al.*, 1944; Rasmussen, Waisman, and Lichstein, 1944; Clark *et al.*, 1945; Lichstein *et al.*, 1945). The present study is directed to the possible influence of thiamine in the resistance of chicks to avian encephalomyelitis, a disease first described by Jones (1932).

EXPERIMENTAL

One-day-old white leghorn chicks maintained in electrically heated cages with raised screen bottoms were used in these experiments. The basal ration was essentially the one described by Briggs *et al.* (1945) and had the following percentage composition: dextrin 60, alcohol-extracted casein 18, gelatin 10, salts 6, and soybean oil 5. Each 100 g also contained *l*-cystine 300 mg, riboflavin 0.6 mg, pyridoxine 0.4 mg, calcium pantothenate 2 mg, niacin 5 mg, choline 150 mg, inositol 100 mg, *p*-aminobenzoic acid 5 mg, biotin 0.02 mg, folic acid 0.10 mg, ascorbic acid 100 mg, 2-methyl-1,4-naphthoquinone 0.05 mg, and α-tocopherol 0.3 mg. In addition, each chick received 3 drops of halibut liver oil (a source of vitamins A and D) weekly.

The strain of virus used was received through the courtesy of Dr. E. Jungherr of the University of Connecticut and was carried through four passages before being used in these experiments. The chicks were given 0.05 ml intracerebrally of either a 1 or 2 per cent suspension of infected chick brain and cord without ether anesthesia.

When placed on the basal ration, 1-day-old chicks usually succumbed within a week to a thiamine deficiency, and since the incubation period for the virus is 10 to 14 days, it was not feasible to use this ration by itself. The addition of 40 μg of thiamine per 100 g of this ration also proved inadequate in extending the survival period, but when the level of thiamine was raised to 60 μg per 100 grams, the chicks survived beyond the virus incubation period, although they showed poor growth and deficiency signs. The chicks were divided into three groups: group 1 received the basal ration containing 60 μg of thiamine per 100 g; group 2, the basal ration containing 90 μg of thiamine per 100 g; and group 3, the basal ration containing 300 μg of thiamine per 100 g. Since the minimum thiamine requirement of the chick is about 150 μg per 100 g of ration, this division allowed study of a deficient, a suboptimum, and an optimum level of the vitamin. The animals were inoculated within a week after they were placed on the diets.

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[1] These studies were aided by a grant from the National Foundation for Infantile Paralysis, Inc. We are indebted to Merck and Company, Rahway, New Jersey, for the crystalline vitamins, and to Wilson and Company, Inc., Chicago, for the gelatin.

The observed signs of this virus disease are a weakness of the legs manifested by an inclination to assume a sitting position. The weakness may progress after a few days to prostration and death. These signs are differentiated from thiamine deficiency by the fact that opisthotonus is absent, that the chicks do not respond to thiamine therapy, and that they do not immediately lose weight. When paralysis occurred in the groups receiving the deficient and suboptimum levels of thiamine, the animals were immediately given, *per os*, 1 mg of thiamine a day for 2 days, in order to determine whether the paralysis was due to the virus infection or to thiamine deficiency.

TABLE 1

The influence of various levels of thiamine on the susceptibility of white leghorn chicks to avian encephalomyelitis

SUPPLEMENT	60 μg B_1 PER 100 GRAMS	90 μg B_1 PER 100 GRAMS	300 μg B_1 PER 100 GRAMS
12 days after inoculation			
No. of chicks without paralysis	5	22	31
No. of chicks paralyzed	30	15	5
22 days after inoculation			
No. of chicks without paralysis	1	8	19
No. of chicks paralyzed	34	29	16

These chicks were inoculated within a week after they were placed on experiment.

In the first series, which included 12 chicks in each group, those which received the largest supplement, 300 μg thiamine per 100 g, exhibited a lower incidence of infection than those in either of the other two groups. The incubation period for the virus was longer, the paralysis was less severe, and fewer deaths occurred in the paralyzed chicks.

This series was repeated twice with similar results in each case; the composite figures are given in table 1. More than 50 per cent of the chicks receiving 300 μg of thiamine per 100 g of ration were protected from the virus infection, compared to practically no protection in the group receiving the 60 μg supplement and 22 per cent in the group receiving the 90 μg supplement.

Another series of experiments was run simultaneously in which 1-day-old chicks were given the basal ration supplemented with 300 μg of thiamine for 2 weeks. Then they were divided into 3 groups to receive the same supplements of thiamine as in the other series (60, 90, and 300 μg per 100 g of ration, respectively). After 2 weeks on this regimen, all chicks were inoculated with the virus.

In this series the chicks maintained on the optimum level of thiamine were not protected, the greatest percentage of surviving chicks being in the group receiving 60 μg of thiamine per 100 g of ration. This experiment was also repeated twice with similar results. The composite results are presented in table 2. It is interesting to note that the incubation period for the virus was reduced in the group receiving the highest level of thiamine, and in some cases paralysis was observed 5 days after inoculation. Only 4 chicks out of 34 failed to show paralysis in this group as compared with 10 out of 27 in the group receiving 60 μg of thiamine per 100 g of ration.

It is evident that the degree of protection afforded the chicks against this virus depended upon a number of factors, which included the age of the chick, the previous state of nutrition, and the state of nutrition at the time of inoculation.

Reports from this laboratory have shown that mice fed diets deficient in thiamine manifest a striking resistance both to Lansing strain poliomyelitis and to Theiler's encephalomyelitis (Rasmussen, Waisman, Elvehjem, and Clark, 1944), but the level of thiamine does not appear to be a crucial factor in the susceptibility of monkeys to the MV strain of poliomyelitis virus (Clark *et al.*, 1945). The results obtained with our first series of chicks, which were started immediately on the depletion diets, showed an entirely different picture from those exhibited either by the mouse or the monkey in that the higher levels of thiamine gave the greatest protection. On the other hand, the results with the chicks that were first given a complete ration and then depleted corresponded more directly with the results in mice. It is possible that the presence of the yolk sac in the younger chicks may have had some influence on the rate of thiamine depletion and hence some effect on the degree of resistance.

TABLE 2

The influence of various levels of thiamine on the susceptibility of white leghorn chicks

SUPPLEMENT	60 μg B_1 PER 100 GRAMS	90 μg B_1 PER 100 GRAMS	300 μg B_1 PER 100 GRAMS
12 days after inoculation			
No. of chicks without paralysis	18	6	12
No. of chicks paralyzed	9	18	23
22 days after inoculation			
No. of chicks without paralysis	10	2	4
No. of chicks paralyzed	17	22	30

These chicks were given a ration with 300 μg of thiamine per 100 grams for 2 weeks, then depleted for 2 weeks, and then inoculated.

SUMMARY

One-day-old white leghorn chicks, divided into 3 groups, receiving a low, suboptimum, and optimum level of thiamine were inoculated with a virus suspension of avian encephalomyelitis. The chicks receiving the highest level of thiamine were protected to the greatest degree.

In another series 1-day-old white leghorn chicks were given an optimum level of thiamine in the ration for 2 weeks, then divided into 3 groups receiving levels of thiamine as indicated in the previous experiments for 2 weeks, at the end of which time they were inoculated. In this case the chicks receiving the lowest level of thiamine were protected to the greatest degree.

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THE ROLE OF SPONTANEOUS VARIANTS IN THE ACQUISITION OF STREPTOMYCIN RESISTANCE BY THE SHIGELLAE[1]

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Recent reports have clearly shown that bacteria tend to become rapidly resistant to streptomycin, both *in vitro* and clinically (Buggs, Bronstein, Hirshfeld, and Pilling, 1946; Miller and Bohnhoff, 1946; Youmans and Feldman, 1946). In the present work we will report on the development of streptomycin resistance in 12 strains of the shigellae and the nature of this *in vitro* resistance. A brief summary of this work has previously been reported (Klein and Kimmelman, 1946).

MATERIALS AND METHODS

Media. Extract broth containing 1 per cent Parke, Davis peptone, 0.3 per cent Difco beef extract, and 0.5 per cent sodium chloride, pH 7.3, was used routinely for all assays.

Streptomycin. Several lots of streptomycin varying in potency from 250 to 500 units per mg were used throughout the work.[2] No difference was observed in the inhibitory action of equivalent units of the several preparations when tested against the shigellae. The streptomycin was diluted in extract broth, and the varying concentrations were assayed in a standard volume of 5 ml.

Test organisms. The 12 strains used in the work are listed in table 1 and are classified according to the schema of Boyd. (For the relationship between this and other classifications see Weil, Black, and Farsetta, 1944.) They were stored on agar slants at 4 C and during the course of the work were transferred once to agar slants.

Inoculum. The standard inoculum in our assays was 0.1 ml of a 20- to 24-hour broth culture containing approximately 200,000,000 bacteria per ml. Titers were read in 48 hours.

EXPERIMENTAL

All 12 test strains were found to be susceptible within the range of 3 to 7 units per ml under the conditions of our assay. The omission of the sodium chloride from the extract broth would have given us higher streptomycin titers. For example, a strain inhibited by 7 units of streptomycin per ml in the presence of 0.5 per cent sodium chloride was inhibited by 2 units per ml in the absence of sodium chloride.

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[1] This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation.

[2] We wish to thank Dr. Chester S. Keefer, Chairman of the Committee on Chemotherapeutic Agents of the National Research Council, and the Wyeth Institute of Applied Biochemistry for our supply of streptomycin.

The rate of the development of streptomycin resistance in these 12 strains was determined by repeated subculturing after 48 hours' incubation at 37 C from the last streptomycin broth tube showing growth in the initial assay to broth tubes containing increasing concentrations of streptomycin. For example, starting with a strain of *Shigella dysenteriae* which was inhibited by 6 units and subculturing 0.1 ml from the growth in 5 units, then subculturing from the growth in 20 units, we obtained growth in 1,000 units per ml after only two subcultures. A large inoculum of resistant bacteria was not necessary to initiate growth: the culture resistant to 1,000 units that was diluted out so that the test inoculum contained only 1 to 10 bacteria grew readily in 1,000 units of streptomycin per ml. One thousand units of streptomycin per ml was the highest concentration routinely used because of a limited supply of streptomycin, though this strain of *S. dysenteriae* was later found to grow in 10,000 units but not 25,000 units of streptomycin per ml.

TABLE 1

Number of transfers in streptomycin broth of a susceptible culture before growth in 1,000 units of streptomycin per ml was obtained

ORGANISM	ASSAY			ORGANISM	ASSAY		
	1	2	3		1	2	3
S. dysenteriae	2	2	3	*S. paradysenteriae*			
S. sonnei	2	5	2	Flexner 4	11*	3	2
S. ambigua (Schmitz)	9	5		Flexner 5	2	3	
S. paradysenteriae				Flexner 6	2	6	2
Flexner 1	2	7	3	Boyd 1	11	4	
Flexner 2	5	3		Boyd 2	11*	3	2
Flexner 3	4	6		Boyd 3	11*	5	

* Growth in 200 units per ml.

In table 1 we have summarized the results obtained with the 12 strains, indicating the number of transfers in streptomycin broth required before growth was obtained in 1,000 units of streptomycin per ml of broth. Each of the strains was assayed at least twice; six of the strains were assayed three times. We found that the strains varied in the rate at which they became resistant, and the same strain on duplicate assays would often show marked differences in the rate at which resistance developed. Though *S. dysenteriae* consistently became resistant at a rapid rate, several of the other strains showed marked differences in the rate at which resistance developed. For example, in one assay the Flexner 4, Boyd 2, and Boyd 3 grew only in 200 units after 11 transfers, but on repeated assays they showed a more rapid rate of development of resistance. Though the rate of development of resistance varied on repeated assays, it was clear that all the strains were capable of becoming rapidly resistant to streptomycin.

Stability of resistant strains. We determined the resistance of the five strains shown in table 2 after 6 months' storage on agar slants at 4 C, during which time they were transferred once to fresh agar slants. All the strains were still resistant to 1,000 units of streptomycin per ml. We then tested the stability of the strains by subculturing them in plain extract broth daily for 50 days and determining the resistance of the strains after 5, 15, 20, 30, and 50 subcultures. After 50 transfers, two of the strains were still resistant to 1,000 units of streptomycin (table 2), whereas the remaining three strains showed a drop in resistance, though all still possesssed a significant degree of resistance.

We also determined the stability of resistance of four strains at a time when they had developed only a moderate degree of resistance. As shown in table 3, three of the strains were initially resistant to 100 units per ml of streptomycin and one strain was resistant to 50 units per ml of streptomycin. After 30 daily subcultures in broth one of the strains was unchanged in its resistance, two strains showed a moderate drop, and one strain reverted back to approximately its original sensitivity. The results therefore indicate that streptomycin resistance is a relatively stable characteristic of the strains studied, though a drop in resistance may occur upon continued transfer in broth. It was observed that streptomycin resistance was usually associated with a reduction in the rate of growth. The development in a resistant strain of a more rapidly growing susceptible variant might result in a reduction in resistance to the level of the susceptible variant.

TABLE 2

Change in resistance of strains of shigellae initially resistant to 1,000 units of streptomycin per ml following daily subcultures in extract broth

NO. DAILY SUBCULTURES	SHIGELLA DYSENTERIAE	SHIGELLA SONNEI	SHIGELLA AMBIGUA	SHIGELLA PARADYSENTERIAE	
				Boyd 2	Flexner 2
5	1,000*	1,000	250	1,000	1,000
15	1,000	1,000	250	1,000	500
20	1,000	1,000	250	250	500
30	1,000	1,000	250	250	250
50	1,000	1,000	250	250	100

* Units of streptomycin per ml.

TABLE 3

Change in resistance of strains of shigellae initially resistant to 100 and 50 units of streptomycin per ml following daily subcultures in extract broth

NO. DAILY SUBCULTURES	SHIGELLA PARADYSENTERIAE			SHIGELLA AMBIGUA
	Flexner 4	Boyd 3	Boyd 1	
0	50*	100	100	100
5	50	100	100	100
10	50	50	100	50
30	50	10	50	50

* Units of streptomycin per ml.

Certain of our resistant strains occasionally showed atypical reactions in Russell's double sugar agar, giving both an acid slant and an acid butt. However, after 5 to 7 transfers in this medium the cultures would revert to the typical alkaline slant and acid butt with no change in resistance. This is of interest in that it shows that during the *in vitro* development of resistance certain changes in the biochemical activity of the bacteria may be associated with the development of resistance in the shigellae, though resistance may be independent of the observed biochemical changes.

Spontaneous occurrence of streptomycin-resistant variants. In considering the nature of streptomycin resistance, it was thought that in the normal growth of a culture a variant might be thrown off that would be capable of growing in a high streptomycin concentration. At the time of the appearance of this variant the culture would show an increased resistance. Since these highly resistant variants would certainly be very few in number, it would be necessary to examine very large numbers of bacteria for the presence of such variants.

TABLE 4

Variation in streptomycin resistance of bacteria in a susceptible culture of Shigella dysenteriae

20-HR. BROTH CULTURE (200,000,000 PER ML) DILUTION IN SALINE	STREPTOMYCIN AGAR (UNITS PER ML)						
	0	0.5	1	2	3	5	7
10^{-1}	+	+	+	+	+	2	0
10^{-2}	+	+	+	+	79	0	0
10^{-3}	+	+	+	+	8	0	0
10^{-4}	+	+	+	+	1	0	0
10^{-5}	+	+	+	456	0	0	0
10^{-6}	210	260	200	38	0	0	0

Pour plates were prepared by adding 1 ml of each bacterial dilution to 18 ml of varying streptomycin concentrations in melted agar. Colony counts determined in 48 hours.

+ = colonies too numerous to count.

We first determined the distribution of resistant bacteria in our standard inoculum of 20,000,000 bacteria. A 20-hour broth culture of *S. dysenteriae* was diluted in saline in a series of tenfold dilutions to 10^{-6} and each dilution was assayed against concentrations of streptomycin in melted agar varying from 0.5 to 7 units of streptomycin per ml. Pour plates were prepared by adding 1 ml of each bacterial dilution to 15 ml of the varying streptomycin dilutions in melted agar, and colony counts were determined after 48 hours' incubation at 37 C. As shown in table 4, at a concentration of 2 units of streptomycin per ml there was a significant reduction in the number of bacteria, and no bacteria were resistant to 7 units of streptomycin per ml. The highest streptomycin agar concentration permitting growth is closely correlated with the broth titers. In a test sample of 20,000,000 bacteria there were therefore no bacteria resistant to 7 units of streptomycin. When, however, we examined approximately 8,000,000,000 bacteria of the susceptible *S. dysenteriae* strain, we were able to isolate bacteria resistant to 1,000 units of streptomycin per ml. We took 400 one-tenth-ml samples from 40 ml of a 24-hour broth culture and inoculated each sample into a broth tube containing 1,000 units of streptomycin per ml. After 48 hours' incubation we obtained growth in five of the broth tubes. By examining a very large number of bacteria we were therefore able to isolate from a susceptible culture a few bacteria showing the same order of resistance as our most resistant strains.

We did duplicate assays on a total of six susceptible strains for the presence of 1,000-unit variants (table 5), and from every culture we were able to isolate variants resistant to 1,000 units of streptomycin per ml. In three of the cultures no resistant variants were obtained on the first assay in which approximately 8,000,000,000 bacteria were examined, but upon repeating the assays 1,000-unit variants were isolated. Assuming that growth in each tube was due to a single resistant bacterium, we have calculated the ratio of resistant to susceptible cells. These ratios are merely approximations since we did not have enough streptomycin to obtain a more accurate figure by repeated assays on each culture.

TABLE 5

Growth of resistant variants in 1,000 units of streptomycin per ml

	NUMBER POSITIVE		RATIO OF RESISTANT VARIANTS TO TOTAL NUMBER OF CELLS
	Assay 1	Assay 2	
Shigella paradysenteriae (Flexner 4)	24	4	1:570,000,000
Shigella dysenteriae	5	2	1:2,300,000,000
Shigella sonnei	1	1	1:8,000,000,000
Shigella paradysenteriae (Boyd 2)	0	3	1:5,300,000,000
Shigella ambigua (Schmitz)	0	2	1:8,000,000,000
Shigella paradysenteriae (Boyd 1)	0	1	1:16,000,000,000

Four hundred broth tubes containing 1,000 units per ml of streptomycin were each seeded with 0.1 ml (20,000,000 bacteria) of the susceptible cultures.

We were interested in determining whether a pure susceptible culture of *S. dysenteriae* could be obtained by the continuous colony selection of susceptible cells. A 24-hour broth culture of the susceptible *S. dysenteriae* was streaked out on agar plates, and 100 colonies were picked, each colony being inoculated into a broth tube and an agar slant. One-tenth-ml samples of each of the 24-hour broth cultures were assayed against 2, 10, and 25 units of streptomycin. Four per cent of the samples grew in 25 units, 12 per cent grew in 10 units, and all the 100 samples grew in 2 units of streptomycin per ml. A susceptible broth culture inhibited by 10 units was selected, and the growth from the paired agar slant which had not been exposed to streptomycin, but which represented the growth from the same colony, was streaked out. Fifty colonies were transferred to 50 broth tubes, and their paired agar slants and the 50 broth tubes were assayed. A susceptible culture was again streaked out, and the selection and testing of 50 colonies was repeated for a total of six assays. On the sixth assay the culture did not grow as a pure susceptible, but 2 per cent of the samples still grew in 25 units, and 4 per cent of the samples grew in 10 units of streptomycin per ml. The development of streptomycin-resistant variants was then a fundamental characteristic of this strain.

Failure of washed resting cells to develop an increased resistance to streptomycin. Though our results have shown that resistant variants arise independently of streptomycin action, it is still possible that streptomycin may be capable of inducing specific cellular changes in the direction of greater resistance. Since resistant variants arise independently of streptomycin action during the growth of a culture, any study of the role of induced resistance would have to be done under conditions in which active cellular division does not occur. We therefore used washed resting cells in order to determine whether streptomycin could directly modify bacteria in the direction of greater streptomycin resistance. In order to test for any increase in the resistance of resting cells it was first necessary to determine whether streptomycin had any action on resting cells. We found that 5 units of streptomycin per ml after 72 hours at 37 C could sterilize a washed cell suspension of *S. dysenteriae*, and that as little as 1 unit of streptomycin per ml was actively germicidal. We therefore set up the following experiment, using *S. dysenteriae* and *S. paradysenteriae* (Flexner 1) as our test strains.

TABLE 6

Failure of washed resting cells of Shigella paradysenteriae (Flexner 1) to show an increase in resistance following 7 days' exposure to streptomycin*

(Original assay: bacterial count, 120,000,000 per ml; streptomycin tolerance, 5 units per ml†)

AFTER EXPOSURE TO		BACTERIAL COUNT PER ML	STREPTOMYCIN TOLERANCE
Temperature	Streptomycin		
	units per ml		*units per ml*
4 C	0	400,000	1‡
4 C	1	16,000	0.5
4 C	3	40	0§
4 C	10	0	0
Room	0	12,000,000	1
Room	1	150,000	1
Room	3	96,000	0.5
Room	10	25,000	0
37 C	0	200,000	1
37 C	1	0	0
37 C	3	0	0
37 C	10	0	0

* Cells were washed three times in distilled water and suspended in final streptomycin concentrations of 1, 3, and 10 units per ml.

† Growth in 5 units; complete inhibition in 6 units.

‡ Growth in 1 unit; complete inhibition in 2 units.

§ No growth in 0.5 unit.

A 20-hour broth culture was centrifuged, and the cells were washed three times in distilled water and suspended in distilled water. The bacterial count and the initial resistance of the test bacterial suspension were determined as shown in table 6. A series of 12 tubes were then prepared containing 10 ml of the distilled water suspension of bacteria. Three of the cultures were kept as untreated controls, and to the remaining 9 tubes streptomycin was added to give final concentrations of 1, 3, and 10 units. The cultures were divided into 3 groups of 4 each. One group was placed in the refrigerator at 4 C, another was kept at room temperature, and the third group was incubated at 37 C. After 7 days, counts were taken on all the cultures to determine whether streptomycin in the test concentration did possess germicidal activity. The results in table 6 show that all the test concentrations of streptomycin show some germicidal activity. The surviving cells were then tested for their resistance by assaying 0.1-ml samples from the test cultures. All the cultures showed a decrease rather than an increase in resistance to streptomycin. The results obtained with *S. dysenteriae* were similar to those shown in table 6 for *S. paradysenteriae* (Flexner 1).

Increased susceptibility of shigellae following exposure to streptomycin. In addition to noting a decrease in the resistance of our washed cells to streptomycin, we also noted that, during the early development of resistance in our growing cultures, subcultures from the growth in a streptomycin tube instead of showing an increased resistance would occasionally show a decreased resistance or fail to grow. It was found in such cases that, though the bacteria were capable of initiating growth in a given concentration, they were really not resistant to that concentration; for upon continued exposure to streptomycin they were gradually killed. Counts done in such broth cultures at 24, 48, and 72 hours showed a marked reduction in the number of cells, frequently terminating in the sterilization of the culture. Associated with this reduction in count was a reduction in resistance to streptomycin. In certain cases, therefore, growth in a streptomycin broth tube, as judged by turbidity, may not necessarily mean that the bacteria are resistant to that concentration.

DISCUSSION

Our results have shown that *in vitro* the development of resistant variants, independent of the direct action of streptomycin, followed by the selection of these variants in an environment containing a high concentration of streptomycin, is a mechanism for the development of streptomycin resistance.

Demerec (1945) has studied the development of resistance of a strain of *Staphylococcus aureus* to penicillin. He found that in a population of bacteria certain cells were resistant to low concentrations of penicillin, and he indicated that by a series of mutations independent of penicillin action the bacteria gradually became resistant. Our results show that, in the case of streptomycin, resistance is also the result of spontaneous variation independent of the action of streptomycin. But in the case of streptomycin we have found by examining a very large number of bacteria that there were present in a susceptible culture certain bacteria resistant to very high concentrations of streptomycin. A high degree of resistance may therefore result from the presence of a single resistant variant. Variants showing a moderate degree of resistance may also occur as indicated by the relatively slow development of resistance in certain of the strains. Therefore the time at which a variant develops and the magnitude of its resistance should determine the rate at which a culture becomes resistant. We did not undertake a quantitative analysis of the correlation between the rate of development of streptomycin resistance and resistant variants because the work would have required large amounts of streptomycin.

Clinically, a situation analogous to our *in vitro* results may perhaps occur. There may be present in an infection a few resistant bacteria, and, as we have indicated, resistant variants are so few in number that they would readily be missed by the usual assay procedure for streptomycin susceptibility. The infecting bacteria would then be called susceptible. Following streptomycin therapy most of the susceptible bacteria would be killed, and, if the body defenses did not destroy the few remaining resistant variants, they would multiply and we would say the culture had become resistant. Actually we might be dealing with a delayed recognition of the fact that the culture initially contained resistant cells. It is also important to note that if one is confronted with resistant variants of the order of magnitude that we have observed, even using the maximum streptomycin concentrations possible, one could not obtain streptomycin levels capable of destroying these bacteria.

The clinical problem of chemotherapeutic resistant strains is obviously of great importance, and, as has been suggested by several workers, we are probably selecting out from our bacterial population strains resistant to our known chemotherapeutic agents. In the case of streptomycin the development of resistant strains is already a major therapeutic problem. It appears to us that the simultaneous use of streptomycin and an additional chemotherapeutic agent may be an effective means for preventing the continued selection of resistant strains.

We have previously shown that in the case of penicillin and the sulfonamides (Klein and Kalter, 1946) an important factor in the observed synergism resulted from the ability of a relatively low concentration of one chemotherapeutic agent to inhibit the small number of bacteria resistant to the other chemotherapeutic agent. In experiments which we will report at a later date we have found that bacteria resistant to streptomycin are still susceptible to penicillin and the sulfonamides, and that *in vitro* a combination of streptomycin with penicillin or sulfonamides, or both, was more effective than either agent alone. An important factor in the synergism was the inhibition of a few bacteria resistant to the test concentration of one chemotherapeutic agent by the added chemotherapeutic agent.

SUMMARY

Twelve strains of shigellae were studied and the rate at which they became resistant to streptomycin *in vitro* was determined. Streptomycin resistance was

found to be a relatively stable characteristic of these strains, though a drop in resistance did occur with some strains.

By examining very large numbers of bacteria it was possible to isolate from a susceptible culture variants showing a very high degree of resistance.

Washed cells exposed to active concentrations of streptomycin for 7 days showed no increase in their resistance.

It is concluded that the *in vitro* development of streptomycin resistance in the shigellae is the result of the occurrence of resistant variants followed by the selection of these variants in a high streptomycin concentration. A single resistant variant is sufficient to cause a high degree of streptomycin resistance.

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STUDIES ON THE RELATIONSHIP BETWEEN BACTERIOPHAGE AND BACTERIAL HOST CELL

I. Adsorption of Phage by Resistant Variants of Staphylococcus[1]

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Phage-resistant bacteria have been reported by most investigators to show no affinity for the phage to which they are resistant, thus failing to adsorb it (Prausnitz and Firle, 1924; d'Herelle, 1926; Burnet, 1929*a*, 1929*b*; Krueger, 1931; Delbrück, 1942; and others). Resistance in such cases could be due to surface differences in the organisms preventing combination of phage and bacterium. There have been some reports of adsorption by resistant bacteria, but the cases are not clear-cut. Applemans (1922) recorded adsorption by both susceptible and resistant bacteria of the same strain. Flu (1923) reported adsorption by phage-resistant, pathogenic intestinal bacteria. Gohs and Jacobsohn (1926) found that resistant Shiga bacilli adsorbed phage as readily as the susceptible strain. Lysogenic organisms did not adsorb the phage. The resistant and lysogenic variants were isolated from secondary growth obtained after lysis of the sensitive bacteria. Heterologous strains, including *Escherichia coli*, Flexner bacilli, *Eberthella typhosa*, and staphylococci, also bound the phage but to a much less degree. Weiss (1927) observed that bacteriophage entered the bodies of resistant Shiga bacilli, remained there inactive, and could be liberated by tryptic digestion. Burnet and Lush (1935) stated that normal *Staphylococcus aureus* strains almost completely insusceptible to a strong phage adsorbed it as readily as a susceptible strain, but that strains with "true induced resistance" failed to adsorb phage. Craigie (1940) observed that variants of type II Vi phage were adsorbed as readily by heterologous V form types of *Eberthella typhosa* as by the homologous type, and could be distinguished only by their selective multiplication on the homologous type of organism. Type II phage produced a much larger number of plaques in the homologous type of organism than on heterologous types, showing that the latter were resistant, though not completely. No detailed experimental evidence concerning the resistance of these strains or the nature of the adsorption, however, was given in any of these cases. A search of the literature has revealed no clear-cut reports of nonlysogenic, stable, resistant bacteria adsorbing phage as readily as the parent susceptible strain.

The resistant variants isolated in this study were found to adsorb phage to the same degree as the parent susceptible strain.

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Isolation of Resistant Variants

The organism chosen for study was a strain of *Staphylococcus aureus*, 515a, obtained from Dr. M. G. Sevag. This strain was highly sensitive to a staphylococcal phage,[2] giving complete clearing in extract broth cultures and on agar plates[3] at 37 C. Several resistant variants were developed from this sensitive parent strain in the following manner: A young culture of the susceptible organisms was allowed to become completely cleared by the phage (in about 3 hours at 37 C), and the incubation was continued for 40 to 48 hours until secondary growth appeared. When this growth was plated out, resistant colonies could be isolated.

In the case of *Escherichia coli* B, in which the resistant culture develops a few hours after lysis of the susceptible organisms, Luria and Delbrück (1943) consider the resistance to be due to a heritable change of the bacterial cell occurring independently of the action of the virus. They stated that the mechanism may be more complex in cases in which the resistant culture develops only several days after lysis of the sensitive bacteria.

We have no evidence concerning the nature of the origin of our resistant variants. The sensitive strain of staphylococcus grows about 3 times slower than *E. coli* B. This might account for the late appearance of the secondary growth, particularly if the growth rate of the resistant organisms is slowed down further in medium previously exhausted by growth of the sensitive strain. Whether the resistance is due to a heritable change occurring independently of the virus action or to a heritable change induced by the virus may have no direct bearing on the present studies.

The resistant variants, in the presence of an excess of phage, showed no clearing in broth after several days, and no plaques on agar plates, either at 25 or 37 C. Tests for lysogenicity proved negative. The tests were conducted by plating supernatants from cultures of the resistant bacteria with sensitive bacteria. If these variants were lysogenic, the supernatants would contain phage and therefore plaques would be formed when the supernatants were plated with sensitive bacteria. In no case were plaques observed.

To confirm the resistance of these variants, bacterial counts were made during growth in the presence and absence of phage. The experiments were set up as follows: Two tubes of extract broth were inoculated with 0.05 ml of a 16-hour culture, giving approximately 6×10^6 bacteria per ml. To one tube was added 0.1 ml phage, giving 3×10^7 phage per ml, or 5 phage particles for each bacterium. The tubes were incubated at 37 C, without aeration,[4] and counts were made by the pour plate method at intervals over a period of 24 hours.

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[2] A polyvalent staphylococcus phage obtained originally from Dr. W. J. MacNeal.

[3] Extract broth medium contained 3 g beef extract (Difco), 10 g peptone (Parke, Davis), and 5 g NaCl per liter. Agar medium contained the same + 1 per cent agar. One per cent agar was used to obtain larger plaques. With 2 per cent agar often no plaques were visible macroscopically because of their extremely small size.

[4] Cultures were not aerated since the sensitive strain was quickly lysed without aeration, and the resistance of the variants was maintained during aeration.

Table I gives counts from three typical experiments. The resistance of the variants has been maintained thus far for 1½ years in spite of constant subculturing.

Adsorption of Phage

Adsorption was determined in the following manner: Three-hour cultures of the susceptible and resistant variants were prepared in extract broth, concentrated by centrifugation, and then diluted with broth to give the desired number of bacteria per ml. The organisms were then mixed with a known amount of phage (5×10^7 particles per ml), and at designated intervals (figure 1) a sample was removed and immediately diluted 1:100 with saline to prevent further adsorption. These diluted samples were then centrifuged for 5 minutes to throw down the bacteria with the adsorbed phage. The supernatants were plated with sensitive bacteria to determine the unadsorbed fraction. In the case of the resistant bacteria, the unadsorbed fraction could be determined without centrifugation, since the phage adsorbed could not produce plaques. Similar results were obtained with and without centrifugation. Figure 1 shows the adsorption of phage by the parent susceptible strain and by three resistant variants for an adsorption period of 10 minutes. In each case the adsorption rate constant[5] of the resistant variants is higher than that of the sensitive variant. Heat-killed organisms of both susceptible and resistant variants also adsorbed phage. The adsorption of phage by the resistant variants is a specific adsorption, since neither coli nor dysentery phage was adsorbed. Likewise, coli and dysentery organisms did not adsorb the staphylococcal phage.

TABLE 1

Growth of resistant variant with and without phage

HOUR	BACTERIAL CONCENTRATION IN MILLIONS PER ML					
	R_c			R_c + phage		
	Expt. 1	2	3	1	2	3
0	6.2	6.0	6.2	6.1	5.9	6.2
1.5	6.9	6.6	6.8	6.5	6.3	7.1
3.0	20.3	17.3	18.5	19.8	14.6	22.6
5.5	175.	123.	129.	150.	112.	135.
7.0	287.	250.	233.	267.	230.	240.
24.0	960.	870.		1,170.	885.	

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[5] The adsorption rate constants were derived from the slope of the straight lines of the plot on a logarithmic scale (Delbrück, 1940).

$K = \dfrac{-\log_{E}\dfrac{P_f}{P_o}}{[B]\,t}$ in which $\dfrac{P_f}{P_o}$ = unadsorbed (free) phage at time t/initial free phage. The straight line relationship holds until about the sixth minute, when 95 to 99 per cent of the phage has been adsorbed. The rate then falls off, leaving a few per cent or fraction of a per cent unattached.

The possibility of a diffusible product of the resistant organism destroying the phage was considered. Supernatants from resistant cultures were tested for such destructive power, but no lowering of the phage titers was observed. Moreover, the organisms adsorbed phage even when washed and suspended in saline.

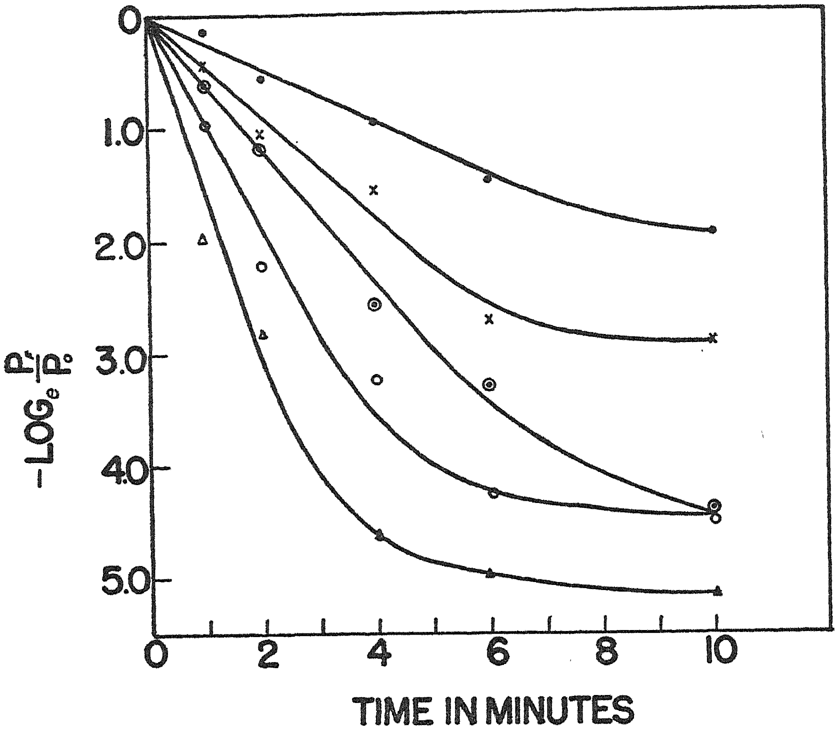

FIG. 1. ADSORPTION OF PHAGE BY S AND R VARIANTS AT 25 C

The bacterial concentrations and the adsorption rate constants derived from the experiments were as follows:

SYMBOL	VARIANT	[B] /ML	$K = \frac{-\log_E \frac{P_f}{P_o}}{[B]\ t}$
●	S	3×10^8	7.7×10^{-10}
○	S	10×10^8	9.5×10^{-10}
+	R_c	3×10^8	14.8×10^{-10}
△	R_c	10×10^8	15.7×10^{-10}
◉*	R_b	4×10^8	14.4×10^{-10}

*Unadsorbed fraction determined without centrifugation.

The adsorption of phage onto the resistant bacteria did not bring about a significant multiplication of the phage, at least under the conditions used in these studies. It is quite possible that under some certain conditions (of substrate, pH, etc.) the adsorbed phage could lyse the organisms and multiply. If these conditions could be found, an important key to the mechanism of resistance and susceptibility might be provided.

To show that there was no significant multiplication of the phage, the following experiment was conducted: Cultures were prepared in the same manner as for the adsorption experiments. In each experiment the bacterial concentration was approximately 2×10^8 per ml. The initial phage concentration is given in table 2 for each experiment. Adsorption of the phage was permitted for 10 minutes, after which time a sample of the adsorption mixture was diluted 1:1,000 with broth, and phage assays were made at 0, 1, 2, and 24 hours. Time 0 corresponds to time immediately following dilution. The experiments were conducted at both 25 and 37 C. Table 2 gives phage assays obtained with the susceptible organisms and with three resistant variants. In the case of the resistant variants it is impossible to obtain a correct assay of the phage increase after 2 hours, since the bacteria continue to multiply and, if any phage is produced, some of it will undoubtedly be adsorbed. Up to 2 hours, under the conditions used, there is little or no multiplication of the bacteria, so no chance of further adsorption.[6] The 24-hour assay is merely an assay of the free phage at this time, the whole suspension being diluted and plated with sensitive bacteria. In all cases there was some slight increase in free phage in the presence of the resistant organisms. This might be due to desorption, or might be easily explained by the presence of a very few susceptible cells. The presence of a

TABLE 2

Multiplication of phage with susceptible and resistant variants at 37 C

HOUR	PHAGE CONCENTRATION IN HUNDREDS PER ML					
	S	S	R_a*	R_b	R_b*	R_c
Before adsorption	500,000	1,250,000	10,000	10,000	5,000	200,000
After adsorption and dilution						
0	450	1,160	2.0	1.3	0.8	20.6
1	21,400	29,000	1.4	0.5	0.5	26.6
2	218,000	540,000	2.5	3.0	0.66	50.0
24	1,900,000	2,960,000	3.2	4.4	1.80	68.0

S = susceptible strain.
R_a, R_b, R_c = resistant variants.
* 25 C.

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[6] In later experiments the assay period was extended to 6 hours by reducing the number of bacteria in the dilution mixture. In these experiments the adsorption mixture contained approximately 8×10^7 bacteria per ml and 8×10^7 phage per ml. After adsorption, a sample of the mixture was diluted 1:10,000 with broth. Under these conditions phage assays could be made up to 6 hours before the resistant cultures showed signs of turbidity. The results here confirm and strengthen the conclusion that adsorption of phage onto the resistant bacteria does not bring about a significant multiplication of the phage. In the case of the sensitive strain, a phage increase of 5,000 to 7,000 times occurred in the 6-hour period, whereas in the case of the resistant variants an increase of 5 to 15 times occurred.

few susceptible cells is to be expected, since no bacterial culture can be entirely homogeneous. If the resistant organisms with phage adsorbed were subcultured 2 or 3 times, all signs of phage disappeared.

DISCUSSION

Although this is apparently one of the very few cases of phage adsorption by resistant variants, it precludes the possibility that adsorption per se is a universally determinant factor in susceptibility. It does not, however, conclusively indicate that some surface phenomenon is not responsible for resistance, since there is no assurance that the adsorptive loci are identical in the susceptible and resistant cells. Some system associated with susceptibility may not be available in the resistant variants, or some "protective" system may be present.

Acknowledgment

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SUMMARY

Resistant variants of a strain of *Staphylococcus aureus* are shown to adsorb phage as readily as the sensitive parent strain. The adsorption does not affect the growth of the resistant cells and does not bring about a significant multiplication of the phage.

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ON THE INCLUSIONS OF HANSENULA ANOMALA

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In the cell of *Hansenula anomala* it is possible to recognize two types of inclusions: lipoid inclusions and volutin granules. They are considered to be reserve materials. This may be said of volutin, which disappears in old cultures, but the evidence for the reserve function of the fatty inclusions rests chiefly on their quantitative sensitivity to the composition of the environment.

Hansenula anomala is especially suitable for studying the functions of inclusions, since it seems not to require nutrilites and grows readily in a medium of the following composition: ammonium sulfate, 0.2 g; glucose, 0.2 g; an equimolar mixture of monopotassium and dipotassium phosphate, 0.23 g; and distilled water, 100 ml. In this medium the yeast reproduces normally and deposits relatively considerable quantities of lipoid inclusions and volutin granules.

Most of the observations reported below were made on aerobic microcultures prepared with cells washed several times with sterile, distilled water. Lipoid inclusions were observed in the living cells, observations thus obtained were confirmed by staining with Sudan black B. Volutin was demonstrated by staining with methylene blue at pH 1.7.

Hansenula anomala grows, but to a limited extent and with considerable difficulty, in distilled water in which a viable cell usually buds several times; the buds separate from the mother cell but remain small. Observation of the living cells and cytochemical study show that the original lipoid inclusions of the mother cell are neither reduced in size nor do they pass into the bud during growth, but that one or more fatty inclusions are formed *de novo* in each bud; these may slowly increase in size after separation of the bud. On the other hand, growth is accompanied by gradual reduction in the volutin content of the mother cell.

In a solution of ammonium sulfate (0.2 g + 100 ml), the picture is qualitatively similar to that observed in distilled water. However, growth is noticeably faster and goes further than in distilled water, and the rate of disappearance of volutin is such that in 4 days at 25 to 28 C about 80 per cent of the cells are volutin-free.

Of considerable interest is the behavior of cells and inclusions in a solution of glucose (0.2 g + 100 ml). In this environment growth is rapid and extensive, so that a loopful of a heavy suspension of washed cells inoculated into 5 ml of the glucose solution produces turbidity within a day at 33 C. The new cells are ellipsoidal, volutin-free, and each contains at least one relatively huge lipoid inclusion; the protoplasm is hyalin. Such cells do not stain with methylene blue at a low pH, and stain only faintly at pH 4 to 7, so that, in these cells, it is possible to observe the nucleus after fixation by heat and mounting in a dilute solution of methylene blue. However, they remain strongly gram-positive. When these cells are washed several times with distilled water, resuspended in distilled water, and incubated aerobically in microculture, the fatty inclusions become gradually smaller and often disappear. Their disappearance is accompanied by growth and by the reappearance of volutin in the form of small granules variable in number. Upon further incubation of the microculture, new lipoid inclusions are formed. During the time these changes take place in the strongly aerobic microculture, there is no noticeable growth, decrease in size of the fatty inclusions, or formation of volutin in the suspension from which the microculture was prepared.

The observations reported above lead to the conclusion that volutin is readily utilizable by *Hansenula anomala* as a source of nitrogen, but with considerable difficulty as a source of energy. This probably means that volutin is hydrolyzed, that *d*-ribose is not utilizable as a source of energy, and that the purine and pyrimidine bases are suitable sources of nitrogen; the energy expended during the limited growth in distilled water may also have been derived from these bases.

It is also obvious that the lipoid inclusions are noticeably utilizable only by volutin-free cells, in an environment devoid of nutrients, and under strongly aerobic conditions, both as a source of energy and a source of nitrogen. There is no evidence that either type of inclusions is used up during active growth in complete nutritive media; on the contrary, the rate of their formation in such media is highest during active growth.

Since under the proper conditions both types of inclusions may be formed in distilled water, and only by growing cells, and since one type may be transformed to the other, they could not be considered reserve material in the sense of Meyer (1912), i.e., materials "stored by the cell at one time so that they will be used up when external nutrients become relatively deficient, or when there is increase in cell growth, cell respiration, etc." They do not seem to be produced by special processes, but as by-products of normal metabolic processes involved in growth, and because of their insolubility, or limited solubility, they precipitate out in the cell. The transformability of one type to the other under the proper conditions suggests that they are by-products of related metabolic processes.

We have not yet investigated the behavior of the inclusions during sporulation.

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FILTERS SUITABLE FOR SEPARATING SOIL BACTERIA FROM BACTERIOPHAGE

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A few years ago two papers from this laboratory (Bottcher and Hofer, 1943; Conn, Bottcher, and Randall, 1945) referred to the use of Berkefeld N or W filters for the separation of bacteriophage from certain soil bacteria. This recommendation was based on preliminary data that was not published with either paper.

There are two reasons why it now seems advisable to publish the data, together with other information which has since accumulated. In the first place, it is known that the technique of cultivating bacteriophage from soil bacteria has been tried in various quarters with only indifferent success, and it is felt that possibly the failure to get good results may lie in the method of filtration employed. In the second place, recent work in this laboratory has shown that difficulties may occur when one is trying to separate bacteria from bacteriophage with American-made filter candles that are supposed to be satisfactory substitutes for the Berkefeld filters. It is felt that the information obtained in comparing the various types of filters may be of use in helping other workers to avoid some of the pitfalls often encountered in the study of bacteriophage.

Three general types of filters were included in the study, the asbestos pad type, the porcelain candle type, and the sintered glass type. Of all but the glass filters, two or three different manufacturers' products were studied and in most instances several grades of filters. During the course of the work, the German filters (Seitz and Berkefeld) became almost unavailable. The following list includes the various filters studied: Filter pads—Seitz EK No. 1, No. 2, No. 3, G.P., S; Ertel No. 6, No. 7, No. 8, No. 9, No. 10. Filter candles, 2½ inch—Berkefeld W, N; Selas No. 02, No. 03; Mandler, medium grade. Glass filter—Buchner type.

At the beginning of the work, before either of the two above-mentioned papers were published, it was presumed that filter pads would prove more satisfactory than the candle type. This was largely because the quantity of material to be filtered was ordinarily very small, seldom over 8 to 10 ml. This a priori preference (which actually proved unjustified) was based on the greater mechanical ease of drawing small amounts of liquid through a filter pad than through a candle. It was found, however, that the mechanical difficulties of getting the last part of the liquid through a candle could be obviated to a large extent with

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[1] Journal paper 670, New York State Agricultural Experiment Station, Cornell University, Geneva, New York, March 8, 1946.

Several laboratory assistants took part in this work, and special credit is due to Elizabeth J. Bottcher and Challiss Randall.

the Berkefeld and Mandler filters by covering the candle with an inverted test tube of such size as to allow a minimum of space between its walls and the candle. By proper agitation it was possible to eliminate enough of the air between test tube and candle so that the only fluid lost was that actually absorbed by the filter. This scheme could not be used in the case of the Selas filters because of the thick metal band in which they are mounted. The only practical method of using the latter type of filter proved to be to place the filter candle with its opening at the top and to filter from the inside out. (Such filters in the accompanying table are denoted by the word "inverted.") These inverted filters presented other difficulties, however.

METHODS

The methods employed for the preparation of bacteriophage for the kinds of bacteria under investigation have been described in detail by Conn, Bottcher, and Randall (1945). Bacteriophage was obtained from various soils by filtration, several of the above-mentioned filters being used. Tests were then made on each of the filtrates to determine whether the filter was making an efficient separation between bacteria and bacteriophage. To determine this point, tests were made on the filtrates as follows: (1) for the presence of bacteriophage in the filtrate (by observing whether clearing occurred either 24 or 48 hours after inoculation); (2) for sterility of the filtrate (by noticing whether growth occurred in uninoculated tubes both at 24 hours and at 4 days); and (3) for the potency of the phage in the filtrate (by diluting the filtrate several times and observing whether clearing occurred upon inoculation).

There were three general types or groups of bacteria used in this study: (1) three or four different strains of the pea nodule organism, *Rhizobium leguminosarum*; (2) *Agrobacterium radiobacter*; (3) 14 different strains of bacteria isolated from soil showing the morphology typical of *Bacterium globiforme*.

RESULTS

The first observation made on this work was that the asbestos pads of the Seitz type did not prove efficient. Several grades were investigated and none of them proved effective in separating bacteria from bacteriophage. Either the filtrates were not sterile, showing that the bacteria had passed through, or the bacteriophage was filtered out wholly or in part. This was an unexpected finding since other workers had reported Seitz filters as very efficient in obtaining phage from other kinds of bacteria.

As a result of these early findings, no further work was done with this type of filter and the remaining investigations were made on the filters of the other types enumerated above. The results are given in table 1.

This table shows first that, out of the three types of porcelain candles used, best results were obtained with the Berkefeld filters; although, even with these the percentage of sterile filtrates obtained was lower than was desired. The Ertel and Selas filters showed a higher degree of sterility but, on the other hand, showed such a low percentage of lysed cultures that it became apparent that

TABLE 1

Efficiency of filters in separating bacteriophage from bacteria

FILTER	NO. OF TESTS MADE	ORGANISMS STUDIED	PER CENT STERILE	PER CENT LYSED IN DILUTION OF			
				10^{-1}	10^{-2}	10^{-3}	10^{-4}
Berkefeld	22	*Rhizobium* *A. radiobacter* "*B. globiforme*"*	36	45	18	13	9
Ertel nos. 6 to 10	8	*Rhizobium* *A. radiobacter*	62	25	—†		
Selas regular	6	"*B. globiforme*"	0	66	—		
Selas "inverted"	16	"*B. globiforme*" *A. radiobacter*	50	31	—		
Sintered glass	16	"*B. globiforme*" *A. radiobacter*	75	50	37	37	18

* "*B. globiforme*" in this table does not indicate a single species, but rather a group of organisms. Included under this heading were 14 separate strains of bacteria all showing the type of morphology characteristic of *Bacterium globiforme* but not all thought to be the same species.

† A dash in this column indicates either that no test was made in the 10^{-2} dilution, because of lack of sterility, or that no lysis occurred in that dilution.

they were holding back a good portion of the bacteriophage. The second point brought out by the table is the greater efficiency of the sintered glass filters. These filters showed 75 per cent sterility with 50 per cent lysis in the first dilution and as high as 18 per cent even in the fourth dilution.

CONCLUSIONS

It is evident from this work that unsatisfactory results obtained in handling bacteriophage may frequently be due to the type of filters employed. Apparently, some of the types of filters (e.g., the asbestos pad type), which have been reported to give the best results with bacteria from milk, do not prove so satisfactory with soil bacteria. Under the conditions employed in this laboratory and with the particular soil bacteria under investigation, none of the American-made porcelain candles gave as good results as did the Berkefeld grades N and W, which are no longer available. The best results of all were secured with filters of sintered glass.

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NOTES ON MONOPHASIC SALMONELLA CULTURES AND THEIR USE IN THE PRODUCTION OF DIAGNOSTIC SERUMS[1]

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One of the greatest difficulties encountered in the production of *Salmonella* diagnostic serums is their preparation from diphasic cultures in such a manner that agglutinins for only the desired phase appear in the serum. Even though great care is exercised in the preparation of the antigen and although the organisms which are injected apparently represent only a single phase, the resultant serum may contain a considerable agglutinin titer for both phases. If the agglutinins for the suppressed phase amount to 10 to 20 per cent of the titer, the serum is unsuitable for diagnostic use unless it is absorbed. Unfortunately, not all of the antigens from which serums must be derived in order to complete a set of diagnostic reagents have been found in monophasic types. Thus, it is sometimes necessary to isolate one phase of a diphasic culture for the production of serum. Therefore, the discovery of monophasic variants of diphasic types has a very practical bearing, particularly when cultures containing antigens hitherto found only in the diphasic state are concerned.

MATERIALS AND METHODS

The present report deals with a study which has covered a period of five years. The variants described were encountered in the identification of more than 10,000 *Salmonella* cultures. When the study of a variant was undertaken, the latter was planted on an agar slant and transferred at monthly intervals for at least one year. It was examined periodically by agglutination to determine whether phase variation had occurred. At the end of this period it was examined by a modification of the Gard technique described by Edwards and Bruner (1942). This consists of inoculating the culture into semisolid agar which contains agglutinating serum for the dominant phase. Phase variation is evidenced by migration through the medium. At the beginning of the study the organism was also planted in semisolid agar containing agglutinating serum and transferred in the same medium at intervals of 1 to 2 weeks. If variation occurred, the phase which made its appearance was isolated and examined. If the phase which appeared was one of the naturally occurring antigens of the genus, it was subjected to the same treatment as the original culture to determine its stability. A number of induced or artificial phases were produced by serum treatment. These resembled the naturally occurring antigens of the genus only slightly, and they were not studied intensively in this work.

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[1] The investigation reported in this paper was conducted in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

A number of the variants and the phases isolated from them were used to prepare agglutinating serums. These serums were then tested for their suitability as diagnostic reagents.

RESULTS

Salmonella paratyphi B var. *java* (IV, V, XII: b–). This monophasic type long has been used for the production of b serum. Seventy-five cultures of this variety were recognized, and of these 25 were studied intensively. When placed in semisolid agar containing b serum, three of the cultures gave rise to 1,2... phases on the first transfer. The remainder either did not vary or produced artificial phases after many transfers in b serum. It is noteworthy that repeated unsuccessful attempts were made again to isolate 1,2... phases from the three above-mentioned cultures. Apparently the isolation of 1,2... phases from Java strains is largely a matter of coincidence. No variation was noted in the 25 cultures when they were transferred on ordinary agar.

The 1,2... phases isolated from the three Java strains remained perfectly stable when transferred on agar. In the presence of 1,2... serum in semisolid medium they began to spread after eight to ten transfers. From the spreading growth, induced phases were isolated which agglutinated neither with b nor with 1,2... serums. Serum prepared from one of the 1,2... phases agglutinated 1,2... phases to 1 to 10,000 but failed to agglutinate b phases above 1 to 100.

In addition to the Java strains, 10 tartrate-negative, monophasic-specific *S. paratyphi* B cultures were recognized. Seven of these were isolated from man, and three were from spider monkeys. All attempts to isolate 1,2... phases from them failed.

Salmonella typhi-murium (IV, V, XII: i–). Three cultures of this type which contained only phase 1 were found. All attempts to isolate phase 2 (1,2,3...) by cultivation in i serum failed. The cultures remained stable when transferred on agar. Serum prepared from one of them agglutinated i phases at 1 to 40,000 but flocculated 1,2,3... phases to only 1 to 200.

One culture having the formula IV, V, XII:–1,2,3... was found. This is probably a nonspecific culture of *S. typhi-murium*, but, since phase 1 was not isolated, its identity is uncertain. Many reports of the nonspecificity of the Binns strains are to be found in the literature, but Edwards (1936) found i components in them and serum prepared from them contained a high titer of i agglutinins.

IV, V, XIIe, h–. The origin of this monophasic variant is doubtful, since phase 2 was never isolated. Fourteen cultures from man, swine, and fowls were studied. The cultures remained perfectly stable in all tests, neither induced phases nor other normal phases could be found. Serum prepared from one of them agglutinated e,h phases to 1 to 10,000. Related e,n... phases were agglutinated at 1 to 1,000 because of the common e factor. Other phases were not agglutinated above 1 to 500. These 14 cultures were among the variants described by Cherry, Barnes, and Edwards (1946).

Salmonella bredeney (I, IV, XXVII, XII: l, v–). Two cultures were studied, one from man and one from a turkey. No other phases could be isolated from them. Serum prepared from one agglutinated l,v phases at 1 to 10,000 but failed to agglutinate 1,7... phases (phase 2 of *S. bredeney*) at 1 to 100.

Salmonella thompson (VI,VII: k–) and *S. thompson* var. *berlin* (VI,VII:–1,5...). The European literature abounds in references to the Berlin variety, but in this country cultures stabilized in phase 1 occurred much more frequently. Fourteen such cultures were studied. When transferred on agar slants they remained stable. When transferred in the presence of k serum, phase 2 appeared occasionally after one transfer. Usually two or three transfers were required. Serum prepared from the k phase of one culture agglutinated the homologous phase at 1 to 10,000 but did not agglutinate 1,5... phases at 1 to 100. The 1,5... phases obtained from the k phases were equally stable and did not change when transferred on agar slants. After two to four transfers in 1,5... serum they reverted to k.

The four Berlin cultures studied were of European origin and were less stable than the specific phases described above. Although they remained in phase 2 when transferred on agar slants, phase 1 appeared in the first transfer in 1,5... serum. That the k phases thus obtained were quite stable was attested by the serum prepared from one of them. The antigen used was prepared from a phase isolated 4 months previously and transferred on agar at monthly intervals. The serum had a titer of 1 to 40,000 for k phases but did not agglutinate 1,5... phases at 1 to 100.

Salmonella cholerae-suis var. *kunzendorf* (VI,VII:–1,5... and VI,VII: c–). This variety is used to designate the cultures of *S. cholerae-suis* which produce H_2S and which are almost invariably monophasic. Among 1,006 representatives of the variety identified in this laboratory, only four occurred naturally in the diphasic state. The usual form of the variety is VI, VII:–1,5... but rarely phase 1 cultures are found in nature. From practically all 1,5... phases it was possible to isolate c phases after one or two transfers in 1,5... serum. When the 1,5... cultures were transferred on agar no variation was noted. Serums were prepared from two 1,5... phases. These agglutinated the homologous phase at 1 to 10,000 and 1 to 20,000, respectively. Neither agglutinated c phases at 1 to 100.

The c phases obtained from the 1,5... cultures with the aid of serum were more resistant to change than were the original forms. Five of these cultures were studied intensively. Two reverted to 1,5... after two transfers in c serum. Two others required four transfers, but the fifth culture never reverted to 1,5... although upon two occasions it was transferred for more than a year in c serum. A serum prepared from the last-mentioned culture agglutinated c phases at 1 to 10,000, but had no effect on 1,5... phases at 1 to 100.

Eleven cultures represented by the formula VI, VII: c– were found. Four of these were isolated from hogs, and the others were found in man, largely from blood cultures. These natural phase 1 cultures were identical with the phase 1 cultures produced from 1,5... cultures by transfer in 1,5... serum. Apparently they represent natural variations from phase 2 cultures. This view is supported by the results of Kristensen and Bojlen (1936) who found a similar culture in the feces of a patient who was simultaneously excreting phase 2 organisms. The stability of the c phases which occur in nature is equal to that of similar cultures obtained by the use of serum. Some of them were changed to 1,5. . . after two to four transfers in serum but others were resistant to change. No variation was noted when the cultures were transferred on agar slants.

Salmonella newport var. *puerto-rico* (VI, VIII: –1,2,3. . .) was described by Kauffmann (1934) as a totally nonspecific variant of *S. newport*. The original culture transferred on agar slants at bimonthly intervals for 10 years remained entirely in phase 2. A serum prepared from it agglutinated the homologous phase at 1 to 40,000 but failed to agglutinate e,h phases (phase 1 of *S. newport*) at 1 to 100.

When the organism was cultivated in 1,2,3. . . serum an e,h phase was isolated from the first transfer. Five colonies isolated from this phase remained constant when transferred on agar. When placed in e,h serum they reverted to 1,2,3. . . after four to eight transfers. Serum prepared from the e,h phase 16 months after its isolation agglutinated the homologous phase at 1 to 10,000 but did not agglutinate 1,2,3. . . phases at 1 to 100. A second culture of this variety recognized during the course of the work behaved in a manner similar to the original strain.

Salmonella javiana (IX, XII: –1,5. . .), like *Salmonella thompson*, is sometimes entirely in phase 2 when isolated. Three such cultures were studied. When transferred on agar slants the cultures remained entirely in phase 2. When placed in semisolid agar containing 1,5. . . serum, phase 1 (1,z_{28}) appeared in the first transfer. The 1,z_{28} phases were also stable when maintained on agar slants. When grown in 1,z_{28} serum they proved somewhat more resistant to change than did the original 1,5. . . phases. Five colonies of one culture reverted to phase 2 on the first transfer in 1,z_{28} serum. The other two cultures yielded colonies which either did not revert to 1,5. . . or were changed only after seven to ten transfers. All the 1,z_{28} phases remained unchanged when transferred on agar slants. A serum prepared from one of the less stable cultures agglutinated 1,z_{28} phases at 1 to 10,000 and 1,5. . . phases at 1 to 500.

Salmonella worthington (I, XIII, XXIII: 1,w–). Two phase 1 variants were found among 108 cultures identified. One of these was examined intensively over a period of years, and no other phase, either natural or induced, was isolated from it. Serums produced from it agglutinated 1,w phases at 1 to 20,000 to 1 to 40,000 but did not agglutinate z phases (phase 2 of *S. worthington*) at 1 to 100. The second culture was stable under ordinary conditions of culture but yielded induced antigens when transferred in 1,w serum.

Salmonella madelia (I, VI, XIV, XXV: y–). The first culture of this type recognized was a variant which was entirely in phase 1. This culture has remained constant over a period of 4 years when transferred on agar slants. When inoculated into semisolid medium containing y serum, about one-half of the tubes yield phase 2 on the first transfer. Serum prepared from phase 1 agglutinated y phases to 1 to 5,000 but did not agglutinate 1,7. . . phases (phase 2 of *S. madelia*) at 1 to 100. The 1,7. . . phase isolated through the use of y serum was more stable than was phase 1. It remained unchanged when transferred on agar slants, and when placed in 1,7... serum it yielded only induced phases after twelve transfers. Serum produced from phase 2 agglutinated 1,7... antigens to 1 to 5,000 but agglutinated y phases to only 1 to 200.

Salmonella minnesota (XXI, XXVI: b). Fourteen phase 1 variants were found. These variants are quite stable and tend to produce induced phases when cultivated in b serum. From the induced phases it is often possible to obtain e,n,x... phases (normal phase 2 of *S. minnesota*). This work was recently described by Edwards and Moran (1946).

XXVIII: y-. Five of these monophasic forms were found among cultures from turkeys. Since phase 2 has not been isolated, it is impossible to say whether they are variants of *Salmonella tel-aviv* (XXVIII: y–e,n, z_{15}...) or *Salmonella pomona* (XXVIII: y–1,7...). They were quite stable when transferred on agar slants or in a medium containing y serum. Like phase 1 of *S. madelia* they should serve excellently for the production of y serum, but as yet they have not been used for that purpose.

DISCUSSION

From the results given above it is evident that monophasic strains of diphasic *Salmonella* types occur not too infrequently. It is also evident that such cultures are excellent antigens for the production of phase-specific serums. Not only is it possible to use the naturally occurring phase to produce serum, but, if the second phase of the culture can be isolated by the Gard method, it also can be used successfully for serum production. This is due to the fact emphasized by Bruner and Edwards (1941) that changing the phase of a monophasic culture does not revive the power of normal phase variation. Thus, the phases which were isolated with the aid of agglutinating serums were just as stable and fixed as were the original antigens of the cultures. In many instances the phases thus isolated were more resistant to change than the antigens from which they were derived. The production of strictly specific c and 1,5... serums from *S. cholerae-suis* var. *kunzendorf* is an excellent example of what may be accomplished with monophasic cultures.

It seems probable that, if persons engaged in *Salmonella* typing examined all monophasic strains carefully, eventually it no longer would be necessary to use naturally diphasic cultures for the production of diagnostic serums.

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SUMMARY

Monophasic variants of a number of *Salmonella* types were described. These furnished excellent antigens for the production of phase-specific serums. When

it was possible to isolate the second phase of these variants by the Gard technique, the phases thus obtained also were useful in the production of specific serums.

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NOTE

A TECHNIQUE FOR MAINTAINING STANDARD PHENOLIC RESISTANCE OF STAPHYLOCOCCUS AUREUS

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The present *in vitro* methods for the evaluation of the germicidal efficiency of quaternary ammonium salts yield varying results. Not only do various workers fail to agree, but individual workers observe erratic, inconsistent results over a period of time. Lack of uniform phenolic resistance of test organisms has been cited as a cause of inconsistent results in the evaluation of germicides. Brewer (Am. J. Pub. Health, **33,** 261) demonstrated that different lots of peptone may alter the resistance of the organisms. Recently Wolf (J. Bact., **49,** 463) prepared a medium which he claims will more uniformly maintain the resistance of *Staphylococcus aureus* than the standard FDA broth. Grubb and Edwards (J. Bact., **51,** 205) reported that incubation of cultures at 40 C "restored and maintained the desired resistance."

We have observed that the use of freshly prepared media is of considerable value in maintaining the standard resistance and the smooth phase of the test organism. It may be that the partial oxygen tension which exists in fresh media is an important factor in this respect.

The ingredients should be obtained in sufficient quantity to assure uniform media over a period of several months or a year. The use of new lots of peptone or beef extract sometimes causes the appearance of rough strains with substandard phenolic resistance. Our stock cultures are sealed with "celons" and stored at 4 C. New broth cultures are made from the stock agar slants once each week, and three daily transfers in broth are made before the culture is used for tests.

By following these suggestions we have consistently retained the standard resistance and the smooth phase of our strain of *Staphylococcus aureus.*

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

THE THEOBALD SMITH SOCIETY (NEW JERSEY BRANCH)

New Brunswick, New Jersey, June 15, 1946

Strepogenin, a New Bacterial and Animal Growth Factor Obtained from Proteins. *Herbert Sprince and D. W. Woolley*, Ortho Research Foundation, Linden, New Jersey.

Evidence was presented that an unknown stimulatory factor is present in Wilson's liver fraction L which effects the same degree of growth response in *Lactobacillus casei*, *Streptococcus faecalis* R, and hemolytic streptococcus, X-40. This factor was called *strepogenin* because of its ability to cause hemolytic streptococci of group A to grow. Trypsin digests of casein and such highly purified proteins as crystalline insulin, chymotrypsinogen, ribonuclease, or tobacco mosaic virus yielded highly active preparations of this material. Other proteins, such as heated, dialyzed egg white, gelatin, or salmine, treated in the same way showed very little activity. Peptic digests were mildly effective, but when these were subjected to trypsin treatment, full release of activity occurred. Strepogenin was not liberated by trypsin from various proteins at the same rate. Generally, maximal liberation occurred at or before 20 hours.

Methods for concentrating strepogenin were mentioned, and its chemical properties were discussed. Recently, strepogenin has been demonstrated by Woolley to increase the rate of growth of mice.

The Amino Acid Nutrition of Streptococcus faecalis. *David Perlman*, Research Laboratories, Merck and Company, Inc., Rahway, New Jersey.

Streptococcus faecalis Rg. 1-A (ATCC-9790) can be adapted to grow on a medium containing ammonia nitrogen as a replacement for amino acid nitrogen. However, slightly better growth is obtained on media containing several amino acids in addition to the ammonia nitrogen source.

The inhibitory effect of indole-3-acetic acid on the growth of this organism is counteracted by either nicotinic acid or tryptophane. This inhibitory effect was not observed on the growth of an organism adapted to utilize ammonia nitrogen.

Decomposition of Marine Paints and Their Paint Constituents in Sea Water. *Robert L. Starkey and John D. Schenone*, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, New Jersey.

The decomposability of marine paints in sea water was tested by measuring their effects on bacterial development. Plate counts of bacterial numbers gave little indication of the extent of decomposition. Determinations of oxygen consumption served as an accurate and sensitive index of the relative rates of decomposition. Three days' incubation at 20 to 24 C was used. Paint films on glass surfaces were placed in glass-stoppered culture vessels, which were completely filled with sea water.

Paints which were kept in sea water for 3 to 4 months continued to undergo bacterial attack during the entire period. Some decomposed five times as rapidly as others. Likewise, paint vehicles and plasticizers varied in decomposability. Toxic materials, included in paints as antifouling agents, when present in sufficient concentration to prevent attachment and growth of fouling organisms, did not prevent the development of bacteria on the paint films and bacterial decomposition of the paint constituents.

Resistant Microorganisms and Their Use for the Classification of Antibacterial Substances. *P. C. Eisman, R. L. Mayer, K. Aronson, and W. S. Marsh*, Research Laboratories, Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

A new antibiotic of undetermined chemical structure may appear, by bacterial spectral analysis, to be similar to another antibiotic of known chemical and biological properties. More extended studies, however, often show that the two antibiotics are dissimilar. Many factors tend to obscure interpretations of spectral analyses made for the purpose of comparing one antibiotic with another. It was therefore desirable to investigate the use of microorganisms made resistant to one of these antibiotics for the purpose of differentiating two apparently similar antibiotics. Such resistant organisms should prove to be as sensitive as the unmodified parent culture to the other antibiotic, if the chemical structures of both antibiotics are distinctly different.

This technique was applied to an unknown antibiotic which resembled streptomycin in regard to spectral analysis. The use of a *Staphylocccus aureus* strain, resistant to streptomycin but found to be sensitive to the unknown substance, established the dissimilarity of the two antibiotics.

STREPTIN, AN ANTIBIOTIC FROM A SPECIES OF STREPTOMYCES. *H. Boyd Woodruff and Jackson W. Foster*, Research Laboratories, Merck and Company, Inc., Rahway, New Jersey.

Soils, which were enriched with killed cells of *Mycobacterium tuberculosis* as a source of waxes and fats, were plated in Long's agar, previously seeded with living cells of *M. tuberculosis*, ATCC 607. Many actinomycetes colonies developed at 30 C. Several were surrounded by clear zones showing inhibition of *M. tuberculosis* after final incubation at 37 C. Several similar antagonists which were isolated resembled *Streptomyces lavendulae* in morphological characteristics, but were closely allied to *Streptomyces reticulus-ruber* in biochemical aspects. The isolates produced up to 150 *Escherichia coli* dilution units of antibacterial activity during 2 days' incubation on a rotary shaker at 30 C. The antagonistic broths showed identical antibiotic spectra, but were significantly different from *S. lavendulae* broth. When compared on the basis of equal activity for *E. coli*, broth of the new isolates was 3 to 10 times as active as an inhibitor for staphylococci and micrococci as were *S. lavendulae* cultures.

A concentrate, prepared by the procedure previously employed for streptothricin, yielded a spectrum identical with that of the broth, but more active against staphylococci and micrococci than a streptothricin or streptomycin concentrate prepared in a similar manner. The new antibiotic has been provisionally named streptin.

DOES INACTIVATION OF AN ANTIBACTERIAL AGENT REVEAL ITS MODE OF ACTION? *R. Donovick*, The Squibb Institute for Medical Research, New Brunswick, New Jersey.

CHEMOTHERAPY OF EXPERIMENTAL TULAREMIA. *S. S. Chapman, L. L. Coriell, S. F. Kowal, W. Nelson, and Cora M. Downs*, Camp Detrick, Frederick, Maryland.

SOME ASPECTS OF BACTERIAL ENZYME CHEMISTRY. *D. E. Green*, Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York.

CONNECTICUT VALLEY BRANCH

SMITH COLLEGE, NORTHAMPTON, MASSACHUSETTS, MAY 4, 1946

THE POTENTIATION OF PENICILLIN OR STREPTOMYCIN ACTION BY CERTAIN ENZYME INHIBITORS. *Henry P. Treffers*, Department of Immunology, Yale University School of Medicine, New Haven, Connecticut.

The inhibiting action of penicillin or of streptomycin on a number of organisms can be greatly increased by the addition of certain substances, some of which are known enzyme inhibitors. Enhancement of antibiotic action has been demonstrated with *Salmonella dysenteriae*, *Salmonella ambigua*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* by means of a quantitative turbidimetric assay of growth. Among substances active in this respect are iodoacetic acid, sodium fluoride, sodium azide, merthiolate, cetyl pyridinium bromide, crystal violet, and mapharsin. The amounts of the inhibitor needed vary from those causing negligible inhibition in the absence of the antibiotic, to those giving 50 per cent inhibition of growth. A number of anomalous effects have been obtained when the relative amounts of antibiotic, inhibitor, and inoculum are varied.

STUDIES ON THE PHYSIOLOGY OF PROTOZOA. *G. W. Kidder.*

The ciliated protozoans of the genus *Tetrahymena* can be grown in pure culture; their amino acid requirements are known; and, with one exception, their essential growth factor requirements are known. All strains of *T. geleii* and *T. vorax* so far studied biochemically have either lost all ability to synthesize, or synthesize at an extremely low rate, the amino acids arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophane, threonine, and valine. *T. vorax* strain V is unable to synthesize adequate amounts of alanine and glycine at 30 C. It appears that the enzymes responsible for their synthesis are blocked reversibly at the increased temperature, for blocked cultures returned to 25 C immediately assume normal reproductive rate.

None of the strains of *Tetrahymena* can synthesize vitamin B_c, but must have this substance (or Williams' "folic acid" or Lederle's "folic acid") supplied exogenously. The *Streptococcus faecalis* R factor can partially substitute for vitamin B_c, but only at high levels. Thymine is without effect.

Purines, especially guanine, and pyrimidines, especially cytidylic acid and uracil, must be supplied exogenously. The biosynthesis by the ciliates of thiamine, riboflavin, biotin, and pantothenic acid has been proved, but that of niacin and pyridoxine has been surmised. In each case, however, the rate is low, and an exogenous source is stimulatory. Inositol and *p*-aminobenzoic acid are synthesized in adequate amounts and at adequate rates for metabolic needs. The role of choline is still in doubt.

STUDIES IN BACTERIAL GENETICS. *Joshua Lederberg*, Childs Memorial Fellow in Medical Research, Osborn Botanical Laboratory, Yale University, New Haven, Connecticut.

Auxanography. A method for the identification of the nutritional requirements of biochemical mutants in bacteria is described. A modification of Beijerinck's "auxanographic method," consists of suspending 10^4 to 10^7 washed cells in a minimal agar plate, incubating for 3 to 6 hours, and then placing small drops of various growth factors on the surface. A stimulatory response is shown by a turbid zone appearing in 2 to 24 hours. The advantages of this technique are: (a) substances difficult to sterilize can be tested; (b) a specific growth response is distinguished from a back mutation to wild type, as the latter appears as discrete colonies; and (c) since there is a gradient in concentration from the drop, all levels are tested, and possible inhibitory effects at certain doses do not mask the response. Several unstable nutritional mutants of *Escherichia coli* have been characterized by this method.

Syntrophism. This is defined as the growth of two distinct biochemical mutants in mixed culture as a result of the ability of each strain to synthesize the growth factor required by the other. It has been found that media whose growth factor content severely limits the growth of the individual mutants will support the optimal growth of a mixed culture of two of them. Mutants blocked at different steps in the synthesis of the same factor show syntrophism, presumably by the interchange of precursors. By counting the colonies that appear on plating into different media the composition of the culture in terms of the different mutants can be determined.

STUDIES ON THE ANTIBIOTIC ACTIVITY OF ACTINOMYCETES. *Paul R. Burkholder*, Osborn Botanical Laboratory, Yale University, New Haven, Connecticut.

The antagonism of 7,369 cultures of actinomycetes isolated from soil has been studied with various kinds of test organisms including gram-positive and gram-negative bacteria, acid-fast bacteria, yeasts, molds, and green algae. In this collection, 1,869 isolates inhibited *Staphylococcus aureus* in agar streak plate tests, 261 inhibited *Escherichia coli*, and 514 showed antagonism against *Candida albicans*. The possible ecological significance of actinomycete antagomism against nitrogen-fixing bacteria and green algae in agricultural soils is indicated by numerous tests with streak plates and shaken culture broths.

STUDIES OF MUTATIONS IN ESCHERICHIA COLI. *R. R. Roepke*, American Cyanamid Company, Stamford, Connecticut.

In a study of mutations to growth-factor-requiring strains of *Escherichia coli*, relatively high yields of mutant cultures have been obtained following *X*-ray treatment of cell suspensions. Some of the factors to be considered in a quantitative study of bacterial mutation by X-rays were discussed.

INDUCED BIOCHEMICAL MUTANTS IN ABSIDIA GLAUCA. *Norman H. Giles, Jr.*, Osborn Botanical Laboratory, Yale University, New Haven, Connecticut.

By utilizing a procedure similar to that originated by Beadle and Tatum for the ascomycete *Neurospora*, biochemical mutants have been produced in the phycomycete *Absidia glauca* by irradiation of sporangiospores with ultraviolet. Such mutants fail to grow in a "minimal" medium (glucose, asparagine, salts) but will grow in a "complete" medium (enriched malt, yeast extract). The requirements of most of the mutants have been identified, and in each such instance the addition of a single known substance is sufficient for normal growth. As in *Neurospora*, the mutant types in *Absidia* fall into three main categories, those requiring: (a) B vitamins—pantothenic acid (196) and *p*-aminobenzoic acid (1,200); (b) amino acids—lysine (2,775), tryptophane (2,828), and histidine (10, 50, 2,790—independent mutants), or (c) nucleic acid components—uracil (167) and adenine (1,643). To date, direct studies of the inheritance of such mutant characters have been unsuccessful because of difficulties in securing zygospore germination.

OTHER ANTIBIOTICS. *George Valley*, Biological Laboratories, Syracuse, New York.

MUTATIONS IN MICROORGANISMS. *E. L. Tatum*, Osborn Botanical Laboratory, Yale University, New Haven, Connecticut.

AMMONIA AS AN INTERMEDIATE IN NITROGEN FIXATION BY AZOTOBACTER[1]

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The view that NH_3 might be an intermediate in biological nitrogen fixation was suggested almost immediately following discovery of this process. As has been emphasized repeatedly by recent commentators (Burk, 1937; Burk and Burris, 1941; Wilson, 1940), however, the evidence in support has been so lacking in specificity and proper control that it approaches irrelevancy. As a result an alternative mechanism based on hydroxylamine as the key intermediate has received increasing attention. This view, advocated mainly by Virtanen (1938), is based on experiments which deal with symbiotic nitrogen fixation brought about by association of leguminous plants and the root nodule bacteria. We have already examined the evidence as it applies to this type of fixation and concluded that most if not all would support equally well the view that ammonia is an intermediate (Burris and Wilson, 1945; Wilson, 1940). Nevertheless, an increasing number of authors, especially of texts, present this proposal as a definitely proved mechanism rather than as a suggested hypothesis. Studies made by us since 1941 with N^{15} as a tracer have provided an increasing number of experimental findings pointing to the significance of ammonia in fixation by the free-living bacterium, *Azotobacter vinelandii*. One of the most critical of these is that extremely small concentrations of the ammonium ion will swiftly and completely suppress assimilation of free nitrogen by *Azotobacter* in contrast to the less rapid and less complete substitution noted when other available fixed compounds of nitrogen are supplied the organism. Although reported initially more than three years ago (Wilson, Hull, and Burris, 1943), only recently have we been able to examine in detail this fundamental shift from fixation to assimilation of combined nitrogen. The results are described in this report.

EXPERIMENTAL

As previously described (Burris, 1942), *Azotobacter vinelandii* was allowed to fix molecular nitrogen exclusively for 17 or 18.5 hours. The cultures were aerated vigorously in a N-free medium. Samples were taken periodically, shortly before the addition of the combined nitrogen, to allow estimation of the rate of fixation. Combined nitrogen (10 ppm) as ammonia or nitrate labeled with 32 atom per cent excess N^{15} was then added. Seventy-ml samples were withdrawn and pipetted directly into 100 ml of boiling 0.8 N sulfuric acid, and the solution was again brought to a boil. The cells were centrifuged, washed, and transferred to

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[1] Supported in part by grants from the Rockefeller Foundation and the Wisconsin Alumni Research Foundation.

Kjeldahl flasks. In the ammonia experiment the cells were hydrolyzed with acid to destroy the cells and to release amide and ammonia nitrogen. These samples were made alkaline, and the ammonia was distilled from them. The residues were subjected to Kjeldahl digestion, as were the cells furnished nitrate. Isotope analyses were performed on N_2 derived from the Kjeldahl digests.

The treatment described ensured that any N^{15} found was present in amino acids or other fixed nitrogen compounds not volatile on alkaline distillation, and could not be attributed to occluded ammonia. Control flasks were included in which 70-ml portions of the culture were boiled in 100 ml of 0.8 N sulfuric acid, after which ammonium or nitrate salts enriched with N^{15} were added. These were treated subsequently as described for the other samples and are designated as *ammonia* and *nitrate controls.*

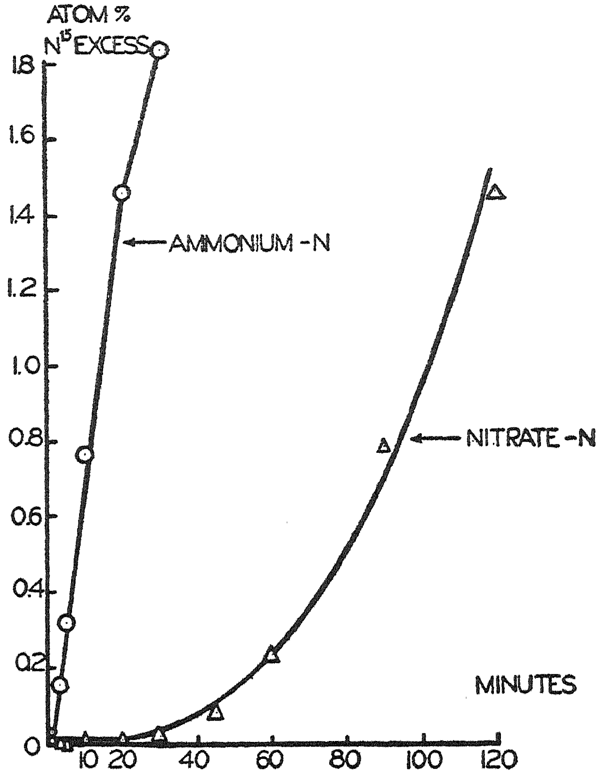

FIG. 1. COMPARISON OF UPTAKE OF AMMONIUM AND NITRATE NITROGEN BY AZOTOBACTER VINELANDII
Organisms previously cultured on N_2; NH_4^+ and NO_3^- controls contained 0.000 atom per cent N^{15} excess

The data from experiments 1 and 2 are recorded in figure 1. They show that, whereas the uptake of ammonia was immediate, a considerable period of adaptation was necessary before the cells could utilize nitrate nitrogen. After 1-minute exposure to ammonia enriched with N^{15} the cells contained 0.031 atom per cent N^{15} excess; this concentration of isotope can be detected readily. The uptake of the ammonia, as evidenced by the content of N^{15} in the cells, was approximately linear for 20 minutes, but the ammonia was exhausted during the 20- to 30-minute interval.

It was not until the 30-minute sample was taken that the quantity of nitrate nitrogen taken up (experiment 2) approached the 1-minute level in the ammonia experiment. In contrast to the linear uptake of ammonia nitrogen, there was an increasing rate of uptake of nitrate nitrogen with time. The initial lag in nitrate utilization and the constantly increasing rate of uptake point to the adaptive formation of enzymes metabolizing the nitrate added and to the previous absence or insufficient concentration of these enzymes. No increased rate of total nitrogen assimilation was observed after the addition of nitrate.

To determine how complete the shift was from molecular nitrogen fixation to ammonia or nitrate utilization reliance must be placed on estimation of the growth rates of the cultures. In experiment 1 the nitrogen content of the culture at 16, 16.5, and 17 hours after inoculation was 13.30, 14.80, and 15.90 mg N per 100 ml; the N^{15} enriched ammonium salt was added at 17 hours and 16 minutes (zero time, figure 1). By plotting the log of mg N per 100 ml against time and extrapolating the line connecting the points at 16.5 and 17 hours, the values of 16.59 mg N per 100 ml at 17 hours and 16 minutes and 17.44 mg N per 100 ml at 17 hours and 36 minutes are observed. Although the logarithmic plot is not strictly linear, as indicated by a slight drop in rate of fixation between the 16.0- to 16.5-hour and the 16.5- to 17.0-hour points, the data, however, furnish a good approximation of the fixation rate.

In the 20-minute period subsequent to ammonia addition at 17 hours and 16 minutes, there was a calculated assimilation of 0.85 mg N per 100 ml; therefore, at the end of this period 4.86 per cent of the nitrogen in the cells had been taken up after the addition of labeled ammonia. Since the ammonium salt supplied contained 32 atom per cent N^{15} excess, the calculated concentration in the cells should have been 1.548 atom per cent N^{15} excess, whereas a concentration of 1.459 atom per cent N^{15} excess was observed. Although these data indicate a small residual fixation in the first 3 minutes after the addition of ammonia (figure 1), in the period from 3 to 20 minutes after the addition, the fixation of molecular nitrogen was almost entirely suppressed. Thus the shift from molecular nitrogen fixation to ammonia utilization was not only very rapid but was virtually complete as well.

That *Azotobacter* cells exhausted ammonia from the medium was indicated by the calculated and observed values for N^{15} content of the cells 30 minutes after the addition of the ammonium salt. The predicted value was 1.822 atom per cent N^{15} excess, and the observed value was 1.837 atom per cent N^{15} excess.

Experiment 2, concerned with the utilization of nitrate, gave distinctly different results. During the 90- to 120-minute period following the addition of nitrate the culture increased in excess concentration of N^{15} from 0.792 to 1.464 atom per cent. Extrapolation of the growth curve for this culture indicated values of 15.37 and 16.47 mg N per 100 ml of culture at the 90- and 120-minute points, respectively. Of the total nitrogen of the cells, 6.68 per cent was assimilated during this period, but analysis for N^{15} showed that only 33.8 per cent of it was derived from the nitrate supplied despite the fact that the cells had had 1.5 to 2.0 hours for adaptation to this source of nitrogen.

To determine the relative utilization of nitrate and ammonia nitrogen by *A. vinelandii* in its early growth stages, ammonium nitrate, with only the ammonium ion enriched with N^{15}, was supplied to cultures in atmospheres essentially devoid of N_2 and containing H_2 to suppress fixation of any residual N_2 present. The cultures were grown in 1-liter, flat-bottomed pyrex bottles with 35 ml of inoculated medium added to a bottle. The 35-ml portions were taken from 500 ml of Burk's 2 per cent sucrose medium (Burk and Lineweaver, 1930) with 286 mg of NH_4NO_3 (100 ppm each of NO_3^--N and NH_4^+-N; only the NH_4^+ was enriched with 32 atom per cent excess N^{15}), inoculated with 15 ml of a 26-hour culture of *A. vinelandii* which had been grown on a medium containing the same concentration of non-enriched NH_4NO_3. A tube containing KOH was supported in each bottle. The bottles were arranged in pairs, and gases were supplied by evacuation and replacement through cotton plugs. At intervals cultures were harvested, and the cells and supernatant medium analyzed for ammonia, nitrate, N^{15}, and total nitrogen. The data are presented in table 1 and figure 2. The last two columns of table 1 are calculated from the atom per cent N^{15} excess in the cells and from the initial values of 28.90 atom per cent N^{15} excess in the NH_4^+ and 0.00 atom per cent N^{15} excess in the NO_3^- of the medium. For example, the cells harvested at 8 hours had 19.62 atom per cent N^{15} excess, which when divided by 28.90 indicates that 67.9 per cent of the cellular nitrogen was derived from NH_4^+ and the rest from NO_3^-.

TABLE 1

Uptake of ammonia and nitrate-N by Azotobacter vinelandii
(Experiment 3)

HR AFTER INOC.	TOTAL N IN CELLS	ATOM % N^{15} EXCESS IN CELLS	NH_4^+-N IN MEDIUM	ATOM % N^{15} EXCESS IN NH_4^+	NO_3^--N IN MEDIUM	% CELL-N FROM ORIGINAL NH_4^+	% CELL-N FROM NO_3^-
0	—	—	7.46	28.90	7.80	—	—
8	2.92	19.62	6.69	24.94	5.49	67.9	32.1
14	9.04	16.67	3.29	13.63	3.08	57.7	42.3
20	13.61	13.91	1.31	9.65	0.62	48.2	51.8
24	14.43	13.48	0.85	7.69	0.26	46.6	53.4

Values for N as mg per 100 ml.
Last two columns calculated from N^{15} analyses.

Although the culture employed had been carried on a medium containing both NH_4^+ and NO_3^-, it utilized ammonium preferentially during its early growth on the medium enriched with N^{15}. Isotopic analysis indicated that two thirds of the nitrogen during the first 8 hours of growth were derived from the ammonium supplied and only a third came from the nitrate. Later, when the ammonium approached exhaustion, the analyses showed that the cellular nitrogen was derived about equally from the nitrate and ammonium ions. The analyses for nitrate and ammonium nitrogen indicated that three times as much nitrate as ammonium disappeared during the first 8 hours, whereas the isotope data showed that two-thirds of the nitrogen assimilated came from the ammonium supplied. To give the N^{15} level observed in the cells, 1.98 mg of ammonia would be required,

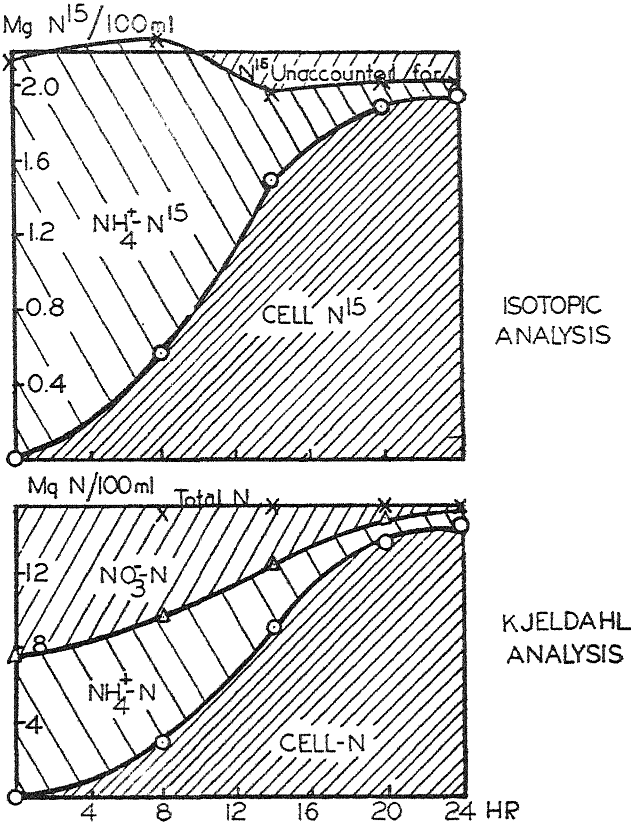

FIG. 2. UPTAKE OF AMMONIUM AND NITRATE NITROGEN BY GROWING CULTURES OF AZOTOBACTER VINELANDII
Organisms previously cultured on KNO_3; supplied NH_4NO_3 (32 atom per cent N^{15} excess in NH_4^+) at time of inoculation

but only 0.77 mg was observed to disappear. This suggested that nitrate was being reduced to ammonia at a considerable rate. The ammonia thus formed diluted the N^{15} of the total ammonium ion present, as is apparent from the values in column 5 of table 1. The isotope analysis, which assumes no dilution, indicated the percentage of cellular nitrogen arising from the *original* NH_4^+ supplied but not from the ammonia derived from nitrate.

Under the conditions of experiment 3 just described it was necessary to wait 8 hours for the first sampling to allow sufficient growth. In experiments 4 and 5, $N^{15}H_4NO_3$ was added to heavy, aerated cultures which could be sampled immediately. In experiment 4, figure 3, a 2-liter culture of *A. vinelandii* was grown with aeration for 18 hours; N_2 was its only source of nitrogen. At 18 hours $N^{15}H_4NO_3$ was added, and aeration continued with a mixture of 4 liters H_2 and 1 liter O_2 per minute. Samples were taken periodically as described in experiment 1, and the NH_4^+-N and NO_3^--N of the supernatant and N^{15} of the cells were determined. As indicated in figure 3, the cells assimilated NH_4^+ immediately, but not

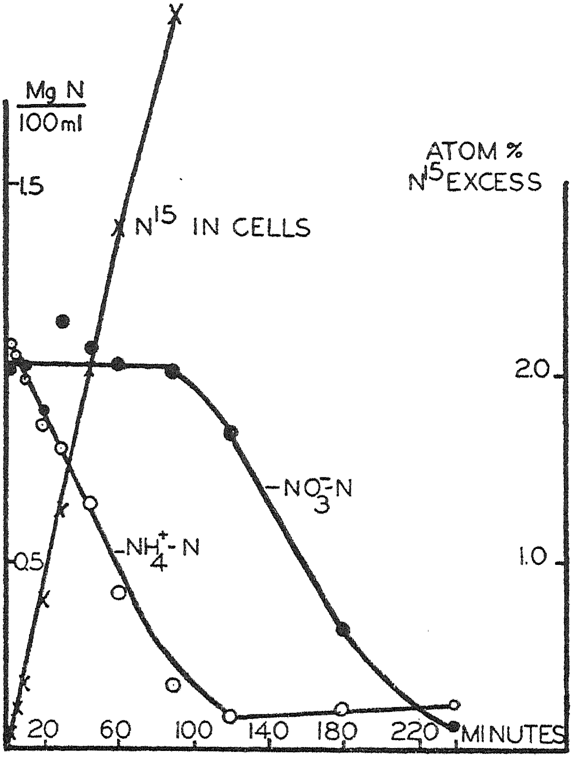

Fig. 3. Preferential Assimilation of Ammonium Nitrogen by Azotobacter vinelandii Previously Grown on N_2
Values for NH_4^+-N and NO_3^--N in both figures 3 and 4 refer to that in the supernatant medium; aerated with H_2, O_2 mixture

until it approached exhaustion did they utilize the NO_3^-. The atom per cent N^{15} excess in the cells likewise indicates a linear uptake of NH_4^+-N. Once assimilation of NO_3^- began, it disappeared at about the same rate as had the NH_4^+. A small residual level of NH_4^+ was present during the later stages of the experiment; this probably came from NO_3^-.

In experiment 5, figure 4, the inoculum of *A. vinelandii* was grown through several transfers with KNO_3 in the medium, and the aerated test culture contained 5 mg NO_3^--N per 100 ml. The culture was aerated for 16.5 hours after inoculation, and then treated with $N^{15}H_4NO_3$ as in experiment 4; it was then aerated with the H_2-O_2 mixture, and sampled at short intervals. This culture which had been acclimated to NO_3^- (most of the KNO_3 added initially had been assimilated when the $N^{15}H_4NO_3$ was added) nevertheless used NH_4^+ in preference to NO_3^-. The shift from N_2 fixation or use of the small amount of residual NO_3^- present to the assimilation of NH_4^+ occurred immediately after the $N^{15}H_4NO_3$ was added. Although the resumption of NO_3^- utilization appeared while somewhat more NH_4^+ remained than in experiment 5, its rate did not seem to be so rapid as that of NH_4^+.

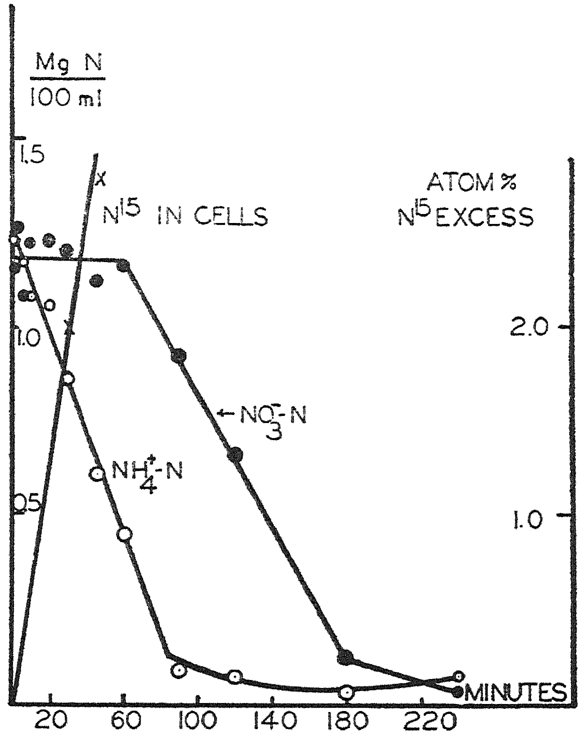

FIG. 4. PREFERENTIAL ASSIMILATION OF AMMONIUM NITROGEN BY AZOTOBACTER VINELANDII PREVIOUSLY GROWN ON KNO_3
(Data of R. MacVicar)

These results establish that when presented with the choice of NH_4^+ or NO_3^- under conditions which preclude N_2 fixation, both in its early and later stages of growth, *A. vinelandii* uses NH_4^+ preferentially. This is true of cultures which have previously fixed N_2 exclusively or have been grown in a medium containing NO_3^-.

SUMMARY

An aerated culture of *Azotobacter vinelandii* grown on N_2 was supplied NH_4^+ labeled with N^{15}; the culture immediately stopped fixing N_2 and used NH_4^+ as its

exclusive source of nitrogen. When N^{15}-NO_3^- was supplied under the same conditions, no N^{15} was found in the cells for 30 minutes in contrast to its detection in 1 minute when N^{15}-NH_4^+ was supplied. The uptake of NH_4^+-N was at a constant rate, whereas the rate of assimilation of NO_3^--N increased with time.

Aerated cultures of *A. vinelandii* previously grown either with N_2 or with KNO_3 assimilated NH_4^+ in preference to NO_3^- when supplied with NH_4NO_3. If NH_4NO_3 was added at the time of inoculation to cultures previously growth with KNO_3, the growing organisms used NH_4^+ preferentially and reduced NO_3^- to NH_4^+.

A. vinelandii grown either with N_2 or NO_3^- has preformed enzyme systems capable of utilizing NH_4^+ immediately and to the exclusion of other nitrogenous compounds. To use NO_3^- the cells must first adapt themselves to it.

From these and other studies made with isotopic nitrogen it is concluded that present information favors the view that NH_3 is the first stable intermediate formed in nitrogen fixation by *Azotobacter*.

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CONCENTRATION OF BRUCELLA SUIS FROM BROTH CULTURE[1]

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The manipulation in the laboratory of comparatively large amounts of pathogenic microorganisms is often complicated by the hazard of infection to which personnel are exposed. This is especially true in such operations as centrifugation, a process which often results in the production of aerosols of the material being spun down. The use of such procedures with highly infective bacteria, e.g., *Brucella* and *Pasteurella*, is, therefore, not encouraged, and the available cell concentrates are limited to those which can be produced and harvested on agar media.

The wartime shortage of agar and acceptable substitutes required that liquid cultures be made as adaptable and productive as possible. In the case of *Brucella suis* this was accomplished by means of aeration in deep liquid cultures. This method has been described in another report (Glassman and Elberg, 1946).

The need for highly concentrated pastes of *Brucella suis* for investigations on airborne infection and for fractionation of antigenic components of the cell was satisfied by a filtration process utilizing ordinary equipment and readily available materials. The method of producing these concentrates also proved useful for other pathogenic organisms and reduced considerably the amount of glassware, labor, and time involved in obtaining bacterial pastes. The hazards of manipulating such materials were almost completely removed, since the separation system was closed except for an efficient air filter.

METHODS AND MATERIALS

The culture of *Brucella suis* was obtained through the courtesy of Dr. I. F. Huddleson and bore the number 1772-A. Cultures of *Brucella abortus* and *Brucella melitensis* were obtained from the Army Medical Center.

The apparatus in use for the filtration process is shown in figure 1. It consists essentially of the cover from a large desiccator jar resting on a 10-inch-diameter Buchner funnel. The Buchner, in turn, is set into a 5-gallon glass carboy through a rubber stopper. A feed tube and rubber stopper drilled with several holes on the side is placed in the desiccator cover so that the broth culture is fed onto the filter bed in small streams. Two air filters composed of Kelly infusion bottles filled with nonabsorbent cotton are placed in the system; one in the desiccator cover to prevent the formation of a vacuum above the filter bed, and another between the vacuum pump and the filtrate reservoir to prevent the dissemination of the organisms in the room atmosphere. The filter bed materials are asbestos and filter-cel.[3]

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[1] Studies conducted at Camp Detrick, Frederick, Maryland, from October, 1944, to May, 1945.

[2] Lieut., USNR, and Major, SnC, respectively.

The apparatus is assembled for use in two parts. One unit consists of the desiccator cover, air filter, terminal part of the siphon system, and the Buchner funnel. A 20-gram bed of asbestos is evenly distributed on the Buchner funnel. The unit is then wrapped and sterilized by autoclaving. The carboy is sterilized separately in the autoclave, and the two units are then joined.

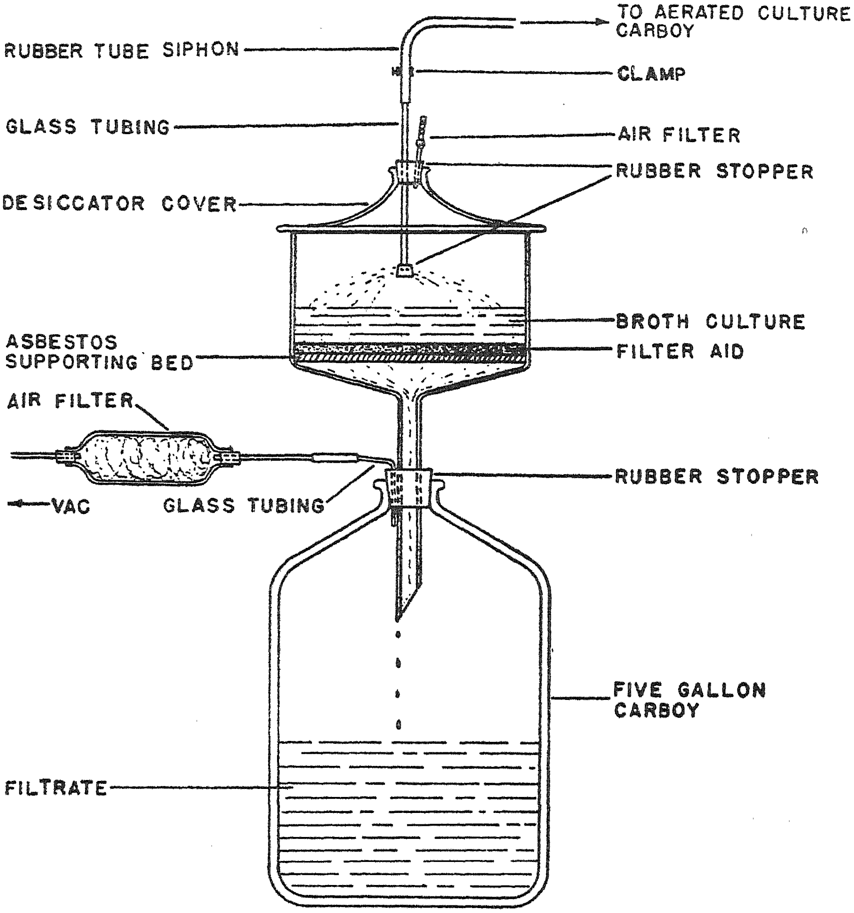

FIG. 1. FILTRATION APPARATUS
Apparatus for large scale laboratory separation and concentration

Seventy grams of filter-cel are sterilized by hot air, suspended in one liter of sterile water, and aseptically poured into the Buchner funnel with care not to disturb the asbestos base. This should be allowed to settle for a few minutes before proceeding. Sterile vaseline is applied aseptically to the edges of the desiccator cover to form an air-tight seal; this may require emery grinding of the rim of the Buchner funnel.

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[3] Filter-cel is a diatomaceous earth preparation obtained from Johns-Manville Company.

The filtration apparatus is attached by means of the siphon system to the culture container and the siphon opened to permit flow of the culture onto the filter. A vacuum of 2 inches of Hg is applied initially to fill the Buchner funnel. When this has occurred, the siphon system is closed and the vacuum increased slowly to match any drop in the rate of filtration. Periodically, the Buchner is refilled from the reservoir of culture. At the end of filtration, the organisms are carefully scraped off the bed by sterile spatulae and collected in any suitable container.

RESULTS AND DISCUSSION

Over a period of several months the filtration method has been used frequently with cultures of *Brucella suis* to give pastes averaging 1×10^{12} cells per gram. It has been found that the bulk of the organisms are located on top of the filter bed. Consequently, they can be obtained with very little of the filter material accompanying them. The small amount of filter-cel which is scraped off can be removed at a later date by resuspension and settling, at which time the filter aid settles very rapidly.

TABLE 1

Ability of Johns-Manville filter-cel to separate Brucella suis from aerated broth cultures

CHARACTERISTICS OF FILTRATION PROCESS	DATA
Weight filter-cel	70 g
Inches vacuum	2–28 inches
Initial count per ml broth filtered	9.0×10^{9}/ml
Volume filtered	5,900 g
Time of filtration	6 hr
Weight of *Brucella suis* harvested	65 g
Count per gram of concentrated *B. suis*	8.3×10^{11}/g
Total number of organisms subjected to filtration	5.3×10^{12}
Total number of organisms harvested	5.3×10^{13}
Concentration factor $\frac{\text{(count per g concentrated } B.\ suis\text{)}}{\text{(count per ml broth (unfiltered))}}$	192

Table 1 gives the data for a typical experiment in which an aerated liquid culture of *Brucella suis* was filtered. The entire yield of organisms in this run was practically confined to the surface of the bed, giving an extremely pure cell paste. Table 2 illustrates the effect of the concentration of organisms in the culture on the rate of filtration. The drop in filtration rate observed when the concentration of organisms in the culture is increased may be ascribed to the formation and packing of the filter cake during the process.

As the filtration process continues, a greater amount of bacterial cake is deposited; and as the cake progressively becomes more tightly packed, the rate of filtration decreases. A heavy suspension of material will build a filter cake in a shorter time on a filtering surface than will a lighter suspension of the same material. As applied to *Brucella suis*, table 2 brings out this point. The amount of negative pressure employed has also been found to effect the over-all flow rate. Contrary to expectation, a high initial negative pressure is not suitable for this type of filtration. A low negative pressure will be found to yield a faster over-all flow rate, presumably because it does not pack the filter cake so tightly as a high negative pressure.

It has been observed that the rate of filtration may be increased by mixing the filter-cel with the culture before the filtration process is begun. The use of the filter aid in this manner is similar to that advocated by the Johns-Manville Company for certain commercial practices. This method does indeed increase the rate of filtration through the formation of a porous filter cake. However, the impurity of the product obtained by use of this method and the lowering of the concentration of the cell paste were two disadvantages which outweighed the time saved by the procedure. Similar results have been obtained with cultures of *Brucella abortus* and *Brucella melitensis*.

TABLE 2

The effect of varying concentrations of Brucella suis in broth cultures on flow rate through filter-cel

TIME OF FILTRATION	VOLUME FILTERED (ML)		
	Water	Culture 1 (3.1 × 10^9 organisms/ml)	Culture 2 (4.9 × 10^{10} organisms/ml)
hr			
½	2,200	2,000	
1		2,750	3,000
1½	6,200		4,000
2	8,500	4,500	
2½	10,500	6,500	
3½	13,500		
4	15,500	8,500	
4½		9,750	
6		10,500	
20			7,000

The virulence of the bacterial pastes described above has been carefully investigated over a long period of time and on many preparations. Graded doses of the concentrates calculated to contain from approximately 2 to 500 cells were injected subcutaneously into groups of 10 guinea pigs. After 30 days the animals were sacrificed and the presence of characteristic lesions in the viscera and sex organs was determined grossly and microscopically. The liver and spleen of each animal were emulsified and cultured on tryptose agar. A fresh tryptose agar transplant of the stock culture under investigation was titrated each time in guinea pigs as a standard for comparison with the bacterial concentrates. The minimum infective dose for 50 per cent of the animals was constantly around 5 organisms for both the standard and concentrated suspensions. No loss in virulence for the guinea pig by the subcutaneous route was encountered.

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SUMMARY

A method for the production of bacterial concentrates from broth cultures of *Brucella* has been described.

Preparations containing 1×10^{12} organisms per gram have been obtained.

The procedure described in this report for concentrating highly infective organisms is less hazardous to workers than centrifugation and yields a bacterial paste which is easily handled.

No loss in the virulence of the cells was encountered as a result of the concentration process described.

REFERENCE

GLASSMAN, H. N., AND ELBERG, S. 1946 The growth of *Brucella* in aerated liquid cultures. J. Bact., **52**, 423–430.

MOTILITY AND SWARMING OF SOME ENTEROBACTERIACEAE

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Reports on the swarming of bacteria are confined almost always to the genus *Proteus*. Such reports always note that with this genus swarming is limited to motile cultures. There must be some property in *Proteus* other than mere motility causing swarming, since actively motile cultures of other genera in the family *Enterobacteriaceae* under ordinary conditions seldom exhibit swarming to any marked degree.

The present investigation started with an observation made in 1938. At this time several hundred stock cultures, including all genera in the family *Enterobacteriaceae*, were carried in unslanted stock agar medium containing 0.75 per cent agar. Inoculations were made by the conventional stab into the center of the medium. All tubes were incubated at room temperature for 2 to 3 days. It was noted that a great majority of the *Proteus* cultures spread completely over the surface of the medium but only an occasional culture showed marked subsurface spreading. On the other hand, cultures of other groups, particularly the paracolon, exhibited marked subsurface spreading with little or no surface spreading.

In 1942 this subject was investigated further. Seventy-five motile *Proteus* cultures, including *P. vulgaris*, *P. mirabilis*, and *P. morganii*, and 75 motile paracolon cultures, including the *Aerobacter*, intermediate, and *Escherichia* sections, were selected. These cultures were carefully inoculated by the stab method into tubes of medium with agar concentrations ranging from 0.4 to 1.1 per cent in increments of 0.1, then 1.5 and 2.0 per cent. All agar used in this work came from one lot of Difco bacto agar, reference number 312155. The greatest concentration of agar was prepared first, and portions were diluted with 1 per cent bacto tryptone broth to give the desired concentration. Evaporation was controlled by weight. Tubes 5 by $\frac{3}{4}$ inches were used. After sterilization, all tubes were held at room temperature for 24 hours, and those with free moisture on the sides of the tube or on the surface of the agar were discarded. Tubes were incubated at room temperature and read after varying time intervals. The greatest extent of surface and subsurface spread was finally recorded on the fifth day. Complete spreading on the surface of the agar and complete subsurface spreading (lateral growth from the line of inoculation to the side of the tube) were recorded as 4. Decreasing degrees of surface and subsurface spreading were recorded as 3, 2, 1, and 0 (no visible spreading).

In the lower concentrations of agar there was little if any difference between the surface and subsurface spreading of the *Proteus* and paracolon cultures. As the agar content of the medium increased, unexpected results were obtained, particularly from the paracolon cultures. On 0.6 to 0.9 per cent agar concentrations, frequently when there was no surface spread, subsurface spreading could be distinguished in a manner wholly analogous to the descriptions of gelatin liquefaction, such as *stratiform, saccate, infundibuliform, crateriform*, and *napiform*. Two additional types were noted, the *umbrella* and *inverted infundibuliform*. In the former type no subsurface spreading occurred 2 to 4 mm beneath the surface of the medium; from this point an *open umbrella* type of spreading was observed. In the *inverted infundibuliform* type, subsurface spread increased with the distance from the surface of the medium, and growth at the bottom extended to the sides of the tube. Table 1 shows the reactions of the different cultures in selected concentrations of agar.

TABLE 1

Surface and subsurface growth of 75 Proteus and 75 paracolon strains

AGAR CONCENTRATION	PROTEUS					PARACOLON				
	Surface: degree of spreading					Surface: degree of spreading				
	4	3	2	1	0	4	3	2	1	0
per cent										
1.0	44*	18	13				3	34	38	
1.5	20	12	33	10				9	46	20
2.0	18	7	25	25				3	42	30
	Subsurface: degree of spreading					Subsurface: degree of spreading				
0.4	74	1				68	6	1		
0.7		17	56	2		10	18	29	18	
0.9				5	70			5	43	27
1.0					75					
1.1									6	69
1.3†									2	
1.5										75

4, 3, 2, 1, 0 = decreasing degrees of spread.

* Number of cultures showing complete (4) surface spread, etc.

† Subsequent work.

In 1 per cent agar 62 *Proteus* cultures exhibited marked surface spreading (reactions of 4 or 3), and 13 cultures showed moderate spreading (reaction of 2). As the concentration of agar increased, surface spreading decreased, although no culture was completely negative even in 2.0 per cent agar. Only 3 paracolon cultures showed marked surface spreading in 1 per cent agar, whereas 30 cultures were completely negative in 2.0 per cent agar. In 0.4 per cent agar all *Proteus* cultures exhibited marked subsurface spreading. Subsurface spreading decreased rapidly as the concentration of agar increased. In a concentration of 0.9 per cent agar only 5 cultures showed any spreading, and at 1.0 per cent all cultures were negative. In 0.5 per cent agar all but one paracolon culture exhibited marked subsurface spreading, in 0.9 per cent 48 cultures showed some spreading, and in 1.1 per cent 6 cultures showed slight spreading. In this work all cultures were negative in 1.5 per cent, but in subsequent work 2 paracolon intermediate cultures exhibited weak subsurface spreading in 1.3 per cent agar.

The 150 cultures were tested for spreading or swarming on 1 per cent agar plates (100 mm) at room temperature. Using the same degrees of reaction as for surface spreading in tubes, 52, 15, and 8 *Proteus* cultures gave reactions of 4, 3, and 2, respectively. One paracolon culture gave a reaction of 4, six a reaction of 2, thirteen a reaction of 1, and 55 were negative.

Stuart *et al.* (manuscript) found that the growth of actively motile strains of an anaerogenic paracolon, type 29911, could be induced to cover completely the surface of a 1 per cent agar plate. These cultures were "conditioned" by serial transfers in tubed semisolid agar, followed by transfers on fresh 1 per cent agar slants, and finally serial transfers on 1 per cent agar plates. Some cultures produced swarming that could not be distinguished from control plates of *P. vulgaris.* To study further the phenomenon of swarming in the *Enterobacteriaceae,* actively motile cultures of 15 *Aerobacter,* 9 intermediate, and 24 *Escherichia* (including normal and paracolon cultures of the 3 sections) were "conditioned" as described. Two cultures were recent isolates, but the remainder were stock cultures 5 to 8 years old. Unless otherwise specified, semisolid agar (0.25 per cent), 1 per cent tubed agar, and 1 per cent plate agar consisted of the desired concentration of agar in 1 per cent bacto tryptone broth. Ten to twelve semisolid tubed agar transfers and a similar number on 1 per cent agar slants were used. Transfers on 1 per cent agar plates were continued as long as spreading or swarming was progressive. Cultures were recorded as positive when the surface of the agar plate was completely covered by the growth of an organism in 48 hours.

Of the 15 *Aerobacter* strains, 8, or 53 per cent, were positive after 4 to 12 transfers on plates. Two strains, recent isolates, swarmed in 4 platings, whereas the other 6 exhibited swarming or spreading or a combination of both in 8 to 12 platings. Of the 9 intermediate strains, 3, or 33 per cent, were positive after 6 to 10 platings, 1 by swarming and the other 2 by a combination of swarming and spreading. None of the 24 *Escherichia* strains was positive. The 7 negative *Aerobacter,* 6 negative intermediate, and most of the *Escherichia* cultures showed some spreading on 1 per cent agar plates. Several of the negative *Aerobacter* and intermediate cultures covered from 50 to 75 per cent of the agar surface, mostly by spreading, though an occasional branch or branches did reach the edge of the plate. An occasional *Escherichia* strain covered as much as 50 to 75 per cent of the agar surface, but usually branches, when present, extended only a few millimeters beyond the edge of the colony.

Early in 1945 certain experiments were undertaken involving several *Salmonella typhi* strains. Two Vi *S. typhi* strains, Bhatnagar and S107, serologically lacked H antigen and in semisolid agar proved to be nonmotile. After serial transfers for 4 to 6 weeks, motile strains were produced. To insure motile strains at all times, these two strains, together with the Watson Vi and H-901 *typhi* strains, have been continuously carried in tubed semisolid agar by weekly transplants from June 2, 1945, to date (June 6, 1946). Since these cultures had been in semisolid agar more than 7 months before the start of the present swarming experiment, they were transferred directly to the 1 per cent agar plates. After several transfers on the 1 per cent agar and bacto tryptone medium, there was no evidence of swarming and the experiment was repeated on 1 per cent agar containing 2 per cent proteose peptone and 0.3 per cent beef extract. By the twelfth transfer on this medium the growth of all strains had reached the maximum extent of spread or swarm. The Bhatnagar and S107 strains covered from 40 to 50 per cent of the agar surface with a relatively thin, unbranched growth. The Watson strain covered about 75 to 80 per cent of the agar surface with a thin film; branches were always present but were so thin as to be almost invisible. Strain H-901 swarmed over the surface of the agar in 48 hours in a manner wholly comparable to control *Proteus* cultures growing on the 1 per cent tryptone agar plates. The results with H-901 were not always consistent. On occasions, of 4 plates inoculated 2 or 3 showed complete swarming, whereas on the remainder about 75 per cent of the agar surface was covered. Many branches reached the edge of the plate without the interstices filling in with growth even after 5 days' incubation.

On June 2, 1945, a strain of typical *Shigella alkalescens* (120) was transplanted to tubed semisolid agar and carried by weekly transfers in this medium until the present, June, 1946. After 6 months in semisolid agar, the first evidence of motility was observed in the form of a small extension of growth outward from the line of inoculation. At this time it was necessary to make two or more transplants from the motile area since often a single transplant might develop only nonmotile forms. Eight months were required to produce a consistently motile strain which covered the surface of the tubed semisolid agar in 24 to 48 hours at room temperature. After 9 months this culture was tested by agglutination and adsorption in antiserum 21811 prepared against a naturally motile strain with the biochemical reactions and the antigenic structure of *S. alkalescens* (Stuart *et al.*, 1943). The flagellar antigens induced on the typical *S. alkalescens* strain 120 were identical with the H antigens of culture 21811.

DISCUSSION

In bacteriological literature "loss variations" are cited much more frequently than "gain variations." Moreover, loss variations often seem to occur spontaneously in more or less freshly isolated cultures. The gain generally must be induced in the parent strain. Twort (1907), for example, induced *S. typhi* to ferment lactose after prolonged cultivation in the presence of this carbohydrate. If gain variations generally must be induced, this may account for the preponderance of loss variations recorded in the literature. Whether gain variations represent the restoration of a lost characteristic or the acquisition of an entirely new characteristic is not known. In either event, however, these variations may constitute a valuable tool in tracing phylogenetic relationships among bacteria, especially the *Enterobacteriaceae.*

The fact that 53 per cent of the *Aerobacter* strains tested exhibited marked swarming or spreading could support Parr's hypothesis (1939) that *Proteus* was derived from *Aerobacter.* As previously pointed out, many *Aerobacter* strains produce urease in varying concentrations (Stuart *et al.*, 1945). Also, an occasional *P. vulgaris* and many *P. mirabilis* strains produce acetylmethylcarbinol (Rustigian and Stuart, 1945). This relationship seems further enhanced by the fact that para-*Aerobacter* 32011 (Stuart *et al.*, 1945), which may attack urea slowly but strongly and which may show small gas volumes, was easily induced to swarm. On the other hand, 33 per cent of the intermediate strains and one *S. typhi* strain were induced to cover completely the surface of 1 per cent agar plates in 48 hours. Of 24 *Escherichia* strains none covered the surface of 1 per cent agar plates.

Gard (1938, 1939) observed swarming by a number of cultures of the *Salmonella* group under certain conditions and concluded that swarming was a sign of motility rather than a property of any particular group of bacteria. We are inclined to agree that actively motile organisms, especially of the *Enterobacteriaceae*, are potential swarmers. Nevertheless, we feel that with few exceptions, under the conditions normally employed in the routine cultivation of bacteria, only the *Proteus* group exhibits marked swarming.

Reports too numerous to cite have shown that rigid lines of demarcation cannot be drawn between certain species and genera in the family *Enterobacteriaceae* on the basis of biochemical reactions and antigenic structure. There is some evidence to indicate that a similar situation is developing with respect to motility. Too often the nonmotile nature of an organism is accepted after one or perhaps a few conventional tests in broth or semisolid mediums.

Shiga (1898) maintained that the dysentery organism he isolated in Japan was motile. Flexner (1901) agreed that dysentery organisms freshly isolated in the Philippines were motile but pointed out that this property was lost on "artificial cultivation." Vedder and Duval (1902) failed to observe motility after prolonged study but did find extensive flagellation in stained preparations of the organisms isolated by Shiga, Flexner, and Kruse. Later Duval and Bassett (1904) by a special technique established a motility in these same organisms that could be "easily distinguished from even the most active Brownian movement." (For a more detailed discussion of this subject see *A System of Bacteriology*, 1929.) From an actively motile *S. alkalescens*-like organism Stuart *et al.* (1943) isolated a weakly motile variant, which, as demonstrated by adsorption experiments, possessed quantitatively as much H antigen as the highly motile parent. Subsequently these same investigators encountered a nonmotile variant of a motile parent. The variant was nonmotile microscopically. In tubed semisolid agar no motile forms were evident, and six serial transfers in semisolid agar plates failed to develop motile forms. Yet the nonmotile variant completely adsorbed H and O agglutinins from an antiserum prepared from the motile parent. A nonmotile paracolon culture was also found that agglutinated to titer with evidence of H agglutination in an antiserum prepared from a motile culture. Upon adsorption the nonmotile culture removed all H and O agglutinins from this antiserum. Twenty serial transfers in semisolid agar plates failed to yield motile forms of the nonmotile strain. Stained preparations of the nonmotile strain, however, revealed the presence of flagella (unpublished data). Comparable findings of nonmotile cultures possessing H antigens and exhibiting flagella in stained preparations have been made in the *Salmonella* group by Edwards of the National Salmonella Center, Kentucky (personal communication).

Stuart *et al.* (manuscript) report one strain of paracolon that required over 2 months of weekly transplants in semisolid agar (0.25 per cent) to induce motility, and another strain which required 5 months for the induction of motility. In the present work, motility was acquired by a perfectly typical strain of *S. alkalescens* in about 6 months, though an additional 3 months were required to produce an actively motile strain. These findings tend to cast doubt on conventional methods for the determination of the ultimate nonmotile nature of a certain strain or perhaps certain species. This suspicion is increased by recent reports of motile organisms with all or at least the major antigens of well-known nonmotile species. Bamforth (1936), Stuart *et al.* (1943), and others have described motile strains with the complete antigens of *S. alkalescens.* Wheeler *et al.* (1946) described two motile strains with the complete antigens and a third with part of the antigens of *Shigella* sp. Q771. E. Hormaeche (personal communication) encountered a motile strain with the complete antigens of *Shigella sonnei,* phase I. P. H. Carpenter (personal communication) found a motile strain with the type-specific antigen of *Shigella dispar.* These findings indicate that nonmotility in the less restricted *Shigella* species may not be so universal a characteristic as has been assumed.

CONCLUSIONS

Although motile members of the family *Enterobacteriaceae,* other than *Proteus,* do not exhibit marked swarming or spreading under ordinary cultural conditions, many may demonstrate this property after proper "conditioning."

By serial transplanting in tubed semisolid agar (0.25 per cent) motility may be induced in certain cultures which are considered to be nonmotile by the usual laboratory tests.

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STUDIES ON THE RELATIONSHIP BETWEEN BACTERIOPHAGE AND BACTERIAL HOST CELL

II. DIFFERENCES IN CARBOHYDRATE METABOLISM OF PHAGE-SUSCEPTIBLE AND PHAGE-RESISTANT VARIANTS OF STAPHYLOCOCCUS[1]

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It is well known that bacterial variants resistant to phage may be developed easily and quickly from a parent susceptible strain, but the reason for the resistance of these organisms is not known. The phage-bacteria relationship resembles in many respects that of the virus and host cell. If the mechanism for phage resistance were known, it might give a clue to the mechanism of phage, and perhaps even of virus, multiplication and point the way for research into the chemotherapy of virus infections.

One possible explanation for resistance is a difference in the surface properties of the two types. In the first report of this series (Henry and Henry, 1946), however, it was found that as far as could be determined there was no difference in phage adsorption by the resistant and susceptible variants used in these studies. Although this is one of the very few cases of phage adsorption by resistant variants, it precludes the possibility that adsorption per se, in this case at least, is the determinant factor in resistance. It does not, however, conclusively indicate that some surface phenomenon is not responsible for resistance, since there is no assurance that the adsorptive loci are identical in the susceptible and resistant cells.

Since phage is dependent on the host cell for multiplication, it seems quite possible that the determinant factor in susceptibility and resistance may depend upon metabolic differences between the two cell types. In view of this a series of investigations was undertaken to determine whether such differences do exist, and in the present report carbohydrate metabolism is surveyed.

The resistant variants under study were isolated as was outlined in the preceding paper (Henry and Henry, 1946). When first isolated, they differed morphologically from the parent susceptible strain only in pigment production, all three resistant variants[2] producing a much more abundant and deeper pigment than the parent. This increased pigment production was not a stable characteristic and varied throughout the course of study, although the resistance was maintained.

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[1] This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation.

[2] Three of the resistant variants isolated (R_a, R_b, R_c) were studied with respect to pigment production, growth, aerobic oxidation of glucose and fructose, and inhibition by urethane, and in no case was a difference observed between variants. In all other studies the variant R_c was used.

Growth. The inoculum for all growth studies was such that the initial concentration was approximately 4 × 10^6 bacteria per ml. Initial growth in all media studied proceeded more slowly in the case of the resistant (R) organisms, although the same point was reached in 24 hours by the susceptible (S) and the R.[3] Growth was followed in extract broth and casein hydrolyzate medium[4] both with glucose (0.5 per cent) and without. Growth in extract broth plus fructose, mannose, and galactose (all 0.5 per cent) was also observed, with the same results. All three R variants responded in like manner. Demerec and Fano (1945) compared the growth rates of 35 resistant mutants of *Escherichia coli* B with the growth rate of the parent strain. On the whole, growth rates for the mutants were lower than for the parent, but no large competitive disadvantage seemed to be systematically associated with phage resistance.

Aerobic oxidation of carbohydrates. With Warburg respirometers, the oxygen consumption by resting cells of the S and R strains with several carbohydrates was determined. The cells were suspended in an M/30 phosphate buffer medium of pH 7.2. Approximately 0.75 mg of organisms (0.3 mg or approximately 9 × 10^8 bacteria per ml in final suspension) were added to each system. The carbohydrate (final concentration = 0.5 per cent) was added from the side arm after a period of equilibration, and the oxygen consumption was read every 15 minutes. The inner cup contained 0.3 ml of 20 per cent KOH to absorb the CO_2. As seen in table 1, the R organisms showed less activity in all cases.

Anaerobic glycolysis. In contrast to the aerobic activity, resting cells of the S and R variants showed no significant difference in their anaerobic utilization of glucose. With fructose and mannose, however, R showed greater activity than S, as is apparent in table 1. The reaction system consisted of 0.75 to 1.0 mg bacteria (0.3 to 0.4 mg or approximately 1 × 10^9 bacteria per ml in final suspension) suspended in M/30 phosphate buffer medium of pH 7.5, 0.2 ml 1 M $NaHCO_3$, and the hexose (0.5 per cent). Air was replaced by 95 per cent N_2 + 5 per cent CO_2.

Carboxylase activity. A study of the dismutation of pyruvate showed that the S strain possessed greater carboxylase activity than the R variants. In four experiments, at the end of the first hour the R variants showed an average of 30 per cent less activity. At the end of the third hour this decreased to 18 per cent and tended to remain constant for at least the two succeeding hours.

Catalase activity. This was measured in Warburg respirometers by the decomposition of H_2O_2 by resting cells. The S and R strains showed the same activity when grown in extract broth with or without 0.5 per cent glucose if the pH was kept at 7 during growth by buffering the medium. However, in ordinary unbuffered extract broth with added glucose, the pH fell to 5 during growth, and here the S strain lost 50 and 70 per cent of its catalase activity in two experiments, but the R strain lost only 16 and 24 per cent.

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[3] Growth in most of the experiments reported was followed turbidimetrically by the Klett-Summerson colorimeter employing a no. 54 green filter. Fifteen suspensions each of S and R, varying in count over the entire working range of all the experiments, were measured turbidimetrically and by viable plate count. The relationship between log number of bacteria per ml and turbidity in Klett units was linear for both S and R, with no significant difference between S and R.

[4] Casein hydrolyzate medium: M/30 phosphate solution (pH 7.4) of 1 per cent casein hydrolyzate (SMACO, vitamin-free) + 7.0 × 10^{-4}M cysteine hydrochloride + 2.0 × 10^{-5}M $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ + 1.8 × 10^{-4}M $MgSO_4 \cdot 7H_2O$ + 1 × 10^{-4}M tryptophane + 1 μg per ml of thiamine chloride and nicotinamide.

Oxidation of p-phenylenediamine. An attempt was made to measure the cytochrome-cytochrome oxidase system by the oxidation of *p*-phenylenediamine. This system is present in *Staphylococcus aureus*, but neither the S nor the R strains could be made to oxidize the diamine either in the respirometer or in spot tests (Sevag and Ross, 1944). The failure of *S. aureus* to oxidize the diamine has been observed before by Sevag and Shelburne (1942). Frei *et al.* (1934) found *S. aureus* to contain cytochromes a, b, and d, but no cytochrome c. In the light of our observations and those of the authors here mentioned, the inability of this organism to oxidize *p*-phenylenediamine may be due to the absence of cytochrome c.

TABLE 1

Aerobic and anaerobic oxidation of carbohydrates by S and R variants

Values represent averages of the number of experiments indicated. All three resistant variants were studied with glucose and fructose, but since no differences were observed, the values were pooled.

CARBOHYDRATE	NO. OF EXPTS.	MEAN QO_2		R < S (%)
		S	R	
Glucose	15	94	64	31.26 ± 8.26*
Fructose	4	96	66	31.4
Mannose	2	94	63	33.0
Galactose	2	23	11	52.2
Pyruvate	4	115	84	27.0
		MEAN QCO_2		S < R (%)
Glucose	5	63	68	7.4†
Fructose	4	21	34	38.2
Mannose	2	27	43	37.2
Galactose	2	0	0	

* Whenever such limits are given, they indicate plus or minus 2 standard deviations. Thus, assuming a normal distribution, only once in twenty should a representative of the particular population of means fall outside these limits as a result of random sampling. The means in this column are means of differences observed in each experiment.

† Insignificant.

Reduction of methylene blue. The glucose dehydrogenase system of resting cells of both strains was studied using the method of methylene blue (MB) reduction in Thunberg tubes. A light suspension of the organism (in phosphate-buffered saline of pH 7.2) was placed in the tube with 0.5 ml of a 0.02 per cent solution of MB. The glucose solution (0.5 per cent = final concentration after mixing) was placed in the side arm; the tubes were evacuated; then the contents of the tube and side arm were mixed after 10 minutes of equilibration in a 40 C water bath. The reduction time was then measured. For at least 3 months after isolation the R variants reduced the MB, with glucose as substrate, in less time than the parent S strain, showing greater glucose dehydrogenase activity. Actually, in 8 experiments, the R variant reduced MB faster than the S strain by 36.4 plus or minus 5.6 per cent. However, when experiments were repeated 7 months after isolation, the difference in reduction time no longer existed, and it appeared that the S and R strains were equal in this activity. In spite of this change, the respective susceptibility and resistance had not altered. This shows that the former difference between R and S in this system was not a determinant in the resistance.

Studies by Use of Inhibitors[5]

The results of the studies of inhibitor effects on the S and R variants are shown in figure 1.

1. Cyanide. Cyanide is an inhibitor of iron-containing enzymes and among respiratory enzyme systems acts specifically on the cytochrome-cytochrome oxidase system.

a. Growth. The medium used was extract broth with and without 0.5 per cent glucose. Neither the difference in inhibition shown between S and R nor the effect of glucose may be regarded as definitely statistically significant as tested by the analysis of variance. However the *t* test (Fisher, 1941), which was more sensitive in this particular case, indicated that R was less susceptible than S by about 8 per cent and that both strains were more susceptible in the presence of glucose by about 13 per cent. There was no consistent change with time over a period of 24 hours.

b. Oxygen consumption. The center well of the Warburg flasks contained a KOH-cyanide mixture as suggested by Krebs (1935).

(1) *Resting bacteria.* The cells (0.3 mg per ml or approximately 9×10^8 bacteria per ml in final suspension) were suspended in M/30 phosphate buffer of pH 7.2, with glucose as substrate. Differences observed between S and R are not significant as determined by the analysis of variance. At the lowest concentration shown on the graph (0.0001 M) there was considerable variation, ranging from 40 per cent inhibition to 25 per cent stimulation.

Although at the highest concentration shown on the graph (0.1 M) the inhibition was 86 per cent for both S and R, the cyanide-stable fraction of total oxygen consumption is taken to be 19.7 plus or minus 1.4 per cent (calculated from 36 values) because of the flat plateau existing over a tenfold increase in concentration and because 0.1 M cyanide is an extremely high concentration for this inhibitor. At this high concentration cyanide may be inhibiting an enzyme which is resistant to it at lower concentrations, or it may be combining with the substrate.

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[5] Most of these experiments studying the effects of various inhibitors were designed and performed on the analysis of variance (Fisher, 1941). The advantages of such design are that with a minimum of replication one has a relatively sensitive statistical test of the significance of the effects of various treatments and variables as well as of their interactions.

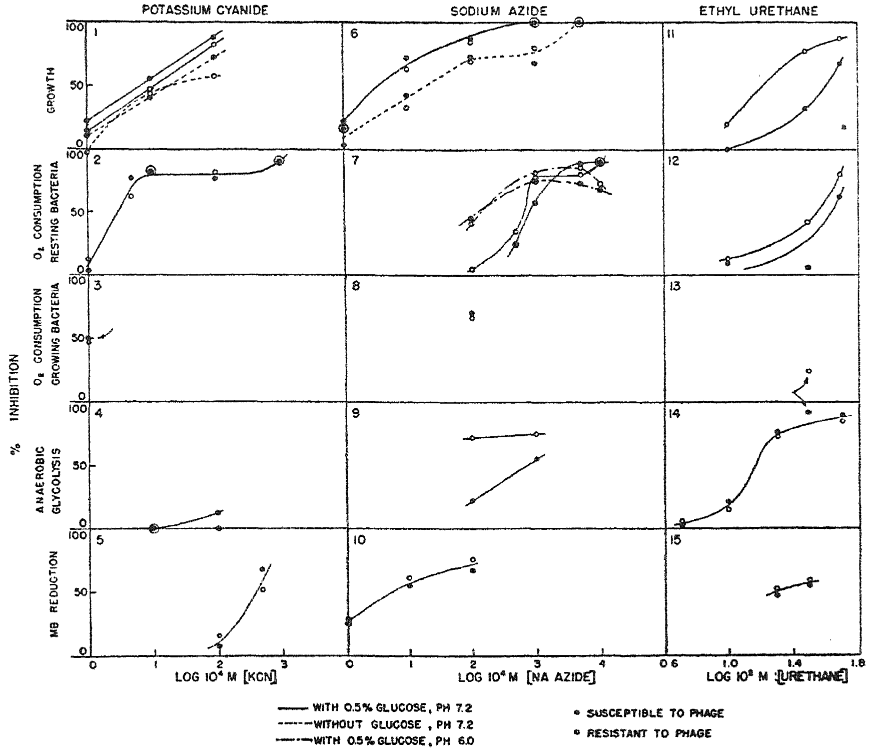

FIG. 1. EFFECTS OF INHIBITORS ON PHAGE-SUSCEPTIBLE AND PHAGE-RESISTANT VARIANTS OF S. AUREUS

1. Each point is the average of 2 experiments measured at 3 and $5\frac{1}{2}$ hours.
2. Each point is the average of three 30-minute periods after the first 20 minutes in 2 experiments (thus the average of 6 values).
3. Only 1 concentration run. Each point is the average of seven 30-minute periods in 2 experiments (thus the average of 14 values).
4. Each point is the average of 3 values.
5. Each point is the average of 5 experiments.
6. Each point is the average of 2 experiments measured at 3, 6, and 24 hours.
7. Each point is the average of 6 values (triplicates measured at second, third, and fourth 30-minute periods).
8. Only 1 concentration run. Each point is the average of the fifth to the eighth 30-minute period in 2 experiments (thus the average of 8 values).
9. Each point is the average of four 30-minute periods in 2 experiments.
10. Each point is the average of 3 experiments.
11. Each point is the average of 8 values (2 experiments run in duplicate, measurements made at 3 and 6 hours).
12. Each point is the average of 12 values (2 experiments run in duplicate, measurements made at second, third, and fourth 30-minute periods).
13. Only 1 concentration run. Each point is the average of 16 values (2 experiments, measurements made at eight 30-minute periods).
14. Each point at the 2 highest concentrations is the average of 2 values, the first and second 30-minute periods. Each point at the 2 lowest concentrations is the average of 8 values (2 experiments, measurements made at four 30-minute periods).
15. Each point is the average of 3 experiments.

Over a period of two hours there was some change in inhibition with time, usually an initial drop followed by a gradual rise.

(2) *Growing bacteria.* In these experiments casein hydrolyzate medium was

substituted for the phosphate buffer, thus enabling the bacteria to multiply. The initial inoculum was 0.3 mg of organisms (0.12 mg or approximately 3.6 × 10^8 bacteria per ml in the final suspension). There was no significant difference between the inhibition of S and R, nor was there any great change in inhibition over a period of 4 hours. The inhibition of oxygen consumption of growing bacteria is calculated on a per cell basis. An interesting observation was made here and in the following studies of oxygen consumption of growing bacteria. In all cases the R variants grew more slowly than the S strain, but the QO_2 of the R organisms was almost invariably higher than that of the S organisms.

c. Anaerobic glycolysis. (For method see page 528.) Cyanide had no effect on either S or R in the range of growth-inhibitory concentrations. The difference between inhibitions of S and R at higher concentrations is merely suggestive.

d. Methylene blue reduction. There was no effect on either S or R in the range of growth-inhibitory concentrations. The difference at higher concentrations is not significant. The percentage of inhibitions of MB reduction are calculated as follows: [1 − (control reduction time/inhibited reduction time)] [100].

2. Azide. It has been supposed that azide acts similarly to cyanide. There have been, however, definite indications to the contrary. Stannard (1939) reported the oxygen consumption of both resting and active frog muscle to be cyanide-sensitive, but only that of active muscle to be azide-sensitive. He interpreted this to mean that there are two cyanide-sensitive systems, one of which is azide-insensitive. Armstrong and Fisher (1940) in a study on the embryonic fish heart reached the same conclusion. Lichstein and Soule (1944) reported staphylococci to be more sensitive to azide in the absence of free oxygen, under which condition heme-containing enzymes are considered to be inactive.

a. Growth. The medium used was extract broth with and without 0.5 per cent glucose, in which 0.5 M azide was needed to inhibit growth completely. The difference between inhibitions of S and R is not significant. Both strains were inhibited more in the presence of glucose.

b. Oxygen consumption

(1) *Resting bacteria.* The analysis of variance showed no significant difference between S and R. Both are more susceptible at a pH of 6 than at a pH of 7.2, indicating that the HN_3 is the active agent (or if required that it must enter the cell to act, that only HN_3 can pass the cell membrane). Others have found this same relationship between activity and pH (Armstrong and Fisher, 1940; Chase, 1942). Azide-stable respiration:

	pH	%	Maximum inhibition at
S	7.2	12	0.5 M
	6.0	25	0.1 M
R	7.2	12	1.0 M
	6.0	15	0.5 M

Inhibition developed within 15 minutes after contact with the azide, following which there was a decrease in inhibition which in turn was followed by a steady rise toward the initial inhibition values. It is an important observation that 70 to 90 per cent inhibition of growth was obtained at a concentration (0.01 M) which scarcely affects the oxygen consumption of resting cells. (In fact, stimulation of respiration frequently occurred at this concentration.)

(2) *Growing bacteria.* Here the inhibition was much greater than with resting bacteria. No inhibition was observed during the first hour, then increasing inhibition during the second hour, approaching a maximum during the third hour. There was no difference between S and R.

c. Anaerobic glycolysis. Inhibition in the case of R was greater than with S. There was no great change with time. This is the only indication of a difference between S and R under anaerobic conditions. The enzyme or enzymes involved here are not identified. In this connection, the effect of azide on carboxylase, with pyruvate as substrate, was studied, and in contrast to the foregoing finding, azide inhibited the carboxylase activity of S more than that of R. In experiments using 0.01 M azide and pyruvate as substrate, at the end of the third hour S was inhibited 98 per cent and R 78 per cent at a pH of 6.0; S was inhibited 55 per cent and R 35 per cent at a pH of 7.0.

d. Methylene blue reduction. Concentrations as low as 0.0001 M inhibited both S and R equally. As with the foregoing instance of inhibition of CO_2 production, the important point here is that the inhibition cannot involve heme systems because they are considered to be inactive under such anaerobic conditions.

3. Urethane. Urethane is one of the so-called "indifferent narcotics" and apparently inhibits flavoprotein enzymes (Quastel, 1943).

a. Growth. The medium used was extract broth with and without 0.5 per cent glucose. The inhibition of R differed significantly from the inhibition of S in an analysis of variance. The inhibition in the case of this inhibitor was not influenced by the presence of glucose. There was usually a slight decrease in inhibition during the first 6 hours of growth and a further decrease in the next 18 hours.

b. Oxygen consumption

(1) *Resting bacteria.* The inhibition of R differed significantly from the inhibition of S in an analysis of variance. The inhibitions fell slightly in the first 30 minutes, then became fairly stable for the next 90 minutes.

(2) *Growing bacteria.* Again R was inhibited significantly more than S. There was considerable change in inhibition with time. Both R and S were inhibited during the first 2 hours, then the inhibition of R increased whereas that of S decreased, gradually reaching stimulation. During the fourth hour the inhibition of R decreased, but only slightly.

c. Anaerobic glycolysis. There was no significant difference between S and R. The inhibition decreased very slightly with time over a period of 2 hours.

d. Methylene blue reduction. During the first 3 months after isolation of the R variants, when they were found to have more glucose dehydrogenase activity than the parent S strain, urethane inhibited the reduction of MB by S more than the reduction by R. The experiments repeated 7 months after isolation showed that the dehydrogenase activities of the two had become equal, and that urethane inhibited the MB reduction the same in both cases. The effect of urethane on growth, oxygen consumption, and anaerobic glycolysis had not changed; the differences observed at first were still present.

4. Sulfathiazole. The sulfonamides form a group of cell inhibitors which have been shown to inhibit numerous enzymes, and although there is some evidence that sulfonamides effect growth inhibition primarily by inhibition of the flavoprotein systems (Sevag and Green, 1944), their mechanism of growth inhibition is still not conclusively known (Henry, 1943).

a. Growth. The medium used here was casein hydrolyzate with and without 0.5 per cent glucose. R was inhibited more than S. Both S and R were inhibited more in the presence of glucose. Table 2 gives the percentage of inhibition as averaged from 5 experiments.

b. Oxygen consumption

(1) *Resting bacteria.* Both S and R were inhibited only about 10 per cent by 0.0066 M sulfathiazole. Approximately 0.75 mg of organisms (0.3 mg or approximately 9 × 10^8 bacteria per ml in final suspension) were added to each system. The medium was M/30 phosphate buffer of pH 7.2, and the substrate was glucose.

TABLE 2

Effect of sulfathiazole on growth of S and R variants

Percentage of inhibition by 0.0066 M sulfathiazole

HOUR	CASEIN HYDROLYZATE		CASEIN HYDROLYZATE + 0.5% GLUCOSE	
	S	R	S	R
3	18	32	33	41
6	20	41	44	64
24	9	34	28	51

Figures are averages of 5 experiments.

(2) *Growing bacteria.* There was a greater inhibition of respiration here, R being inhibited more than S. Inhibition was observed at the first 30-minute reading. In an average of two experiments, S was inhibited 13 per cent and R 31 per cent during the first 2 hours. Between the second and fourth hours stimulation of both respiration and division developed. The medium here was casein hydrolyzate, and the initial inoculum was 0.3 mg organisms (0.12 mg or approximately 3.6 × 10^8 bacteria per ml in the final suspension).

c. Anaerobic glycolysis. There was no inhibition in either S or R by 0.0066 M sulfathiazole.

d. Methylene blue reduction. Sulfathiazole in a concentration of 0.004 M caused acceleration (as much as 65 per cent) of MB reduction by both S and R. Sulfanilamide, 0.04 M, on the other hand, increased the reduction time by both S and R by about 17 per cent (average of 6 experiments).

Attempts to Reverse Susceptibility and Resistance by Use of Inhibitors

If the presence of some particular system in either the S or R strains accounts for their susceptibility or resistance, then by inhibiting this system, the characteristic may be lost. In view of this, the four inhibitors, azide, cyanide, urethane, and sulfathiazole, were added in concentrations giving growth inhibitions of from 20 to 80 per cent, and each strain was observed for changes. To 2-hour broth cultures of the bacteria the proper amount of inhibitor was added, and the tubes were incubated for 2 to 3 hours, allowing growth in the presence of the inhibitor. After this time the phage was added in excess. Under all conditions the S strain retained its high degree of susceptibility and the R variants remained resistant. This work was done aerobically only. It was found, however, that both S and R strains grew well under anaerobic conditions and retained their resistance and susceptibility.

DISCUSSION

The phage-resistant variants utilized in this study were less active than the parent sensitive strain. This observation has been made in other cases of resistance developed toward phage (Anderson, 1944; Demerec and Fano, 1945) and toward other agents including sulfonamides (Sevag, 1946; Yegian *et al.*, 1946), penicillin (Rake *et al.*, 1944), streptomycin (Klein and Kimmelman, 1946) and pyrithiamine (Woolley, 1944). This lower level of activity was evidenced by slower growth rates, decreased aerobic oxidation of sugars, and a decreased dismutation of pyruvate (anaerobic), the latter observation indicating less carboxylase activity. On the other hand, anaerobic glycolysis of fructose and mannose was greater in the R variants. Ethyl urethane was found to inhibit the growth and oxygen consumption of the R variants to a greater degree than the growth and oxygen consumption of the parent S strain, but the enzyme or enzymes involved in the inhibition are unknown in this case. A direct study of the glucose dehydrogenase activity in Thunberg tubes showed in the earlier experiments that R possessed greater activity than S and was inhibited less than S by urethane. In later experiments, however, this differentiation was lost: S and R possessed equal glucose dehydrogenase activity and were equally inhibited by urethane in the Thunberg tube, although the former differences in growth and oxygen consumption inhibition persisted. The fact that in the earlier stages of this study the R variants did reduce MB at greater velocities than the S strain but suddenly lost this differentiation can only serve to emphasize once again the general instability of bacteria, and to make one cautious in concluding that a causative relationship exists between two variables between which there is a correlation.

Sulfathiazole was also found to inhibit the growth and oxygen consumption of growing cells of the R variants more than the growth and oxygen consumption of the S strain, but again the mechanism responsible for this difference is not known.

Approximately 20 per cent of the total oxygen consumption was found to be insensitive to cyanide. Some of this cyanide-stable respiration is apparently connected with growth, because concentrations of cyanide giving maximum inhibition of respiration fail to produce complete growth inhibition. Since growth of both S and R variants is inhibited more in the presence of glucose, it is strongly suggested that a considerable portion of the energy derived from glucose oxidation is utilized by growth processes.

Only 12 per cent of the total oxygen consumption was insensitive to azide, indicating that azide interferes with reactions that are not cyanide-sensitive. It is interesting that at pH 6.0 this insensitive fraction was approximately doubled. As already stated, there have been numerous indications that azide and cyanide behave very differently, and a conclusive example has been found in this particular study. Thus, cyanide was found to inhibit anaerobic glycolysis and MB reduction (glucose substrate) only in concentrations producing maximum inhibition of growth and oxygen consumption. Such an inhibition may result most likely from a combination of cyanide with substrate. Azide, on the contrary, inhibited anaerobic glycolysis and MB reduction at concentrations *much lower* than those required for inhibition of oxygen consumption. It is concluded, therefore, that here azide is a very powerful and rather specific inhibitor of a nonheme enzyme. Since no significant differences were observed in inhibitions of S and R variants with cyanide, it is concluded that resistance is not associated with the cytochrome-cytochrome oxidase systems. The only difference observed in inhibition of S and R by azide was that of anaerobic glycolysis, in which R was inhibited more than S. The enzymes involved in this difference are likewise not clear. The reduction of MB with glucose as substrate was inhibited by azide equally in both S and R. Carboxylase (pyruvate = substrate) was inhibited more in the case of S; so it would appear that neither of these enzymes is responsible for this difference. The various enzymes, however, cannot be assumed to act in the same manner in a chain reaction as they do in isolated systems; therefore results obtained in a study of these isolated systems cannot be carried over and assumed to apply to reactions in which many enzymes are at work in the actual growth and respiration processes.

SUMMARY

Three phage-resistant variants were isolated from a parent susceptible strain of *Staphylococcus aureus*, and a survey was made of the carbohydrate metabolism in an attempt to locate metabolic differences which might be associated with resistance and susceptibility.

The resistant variants had slower rates of growth, aerobic oxidation of sugars, and dismutation of pyruvate. Anaerobic glycolysis of fructose and mannose, however, was greater in the resistant organisms.

A study of the effects of various inhibitors showed significant differences in the case of three of the four inhibitors used, namely ethyl urethane, sodium azide, and sulfathiazole. Ethyl urethane inhibited the growth and oxygen

consumption of both resting and growing cells of the R variants to a greater degree than it did the growth and oxygen consumption of the S strain. Azide inhibited anaerobic glycolysis by the R variants more than that by the S variant. Sulfathiazole inhibited the growth and oxygen consumption of growing cells more in the case of the R variants. In none of these instances can the cause of the difference be defined; the enzyme or enzymes involved are not identified. The resistant variants, with their lower level of activity, undoubtedly lack certain properties or systems possessed by the S variants, and are less versatile in making use of the various metabolic pathways.

The addition of these four inhibitors independently in concentrations giving partial inhibition of growth failed to alter sensitivity and resistance to phage of the S and R variants.

No evidence was obtained to indicate a direct relationship between any of the differences found between resistant and susceptible variants and resistance and susceptibility.

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A STUDY ON THE MECHANISM OF ACTION OF PENICILLIN AS SHOWN BY ITS EFFECT ON BACTERIAL MORPHOLOGY

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Although the exact mechanism by which penicillin exerts its antibacterial effect is not clear, some light may be thrown on this fundamental problem by a consideration of the alterations that it produces in the morphology of susceptible bacteria.

Gardner (1940) was the first to point out the striking changes in shape of bacteria after exposure to varying concentrations of penicillin. *Clostridium welchii*, *Eberthella typhosa*, *Vibrio cholerae*, and even *Escherichia coli* all showed elongation and swelling of the bacterial cells. Staphylococci became spherically enlarged, and streptococci showed both enlargement of the cells and increased length of the chains. It was his interpretation that growth proceeds in the bacteria but that there is failure of fission, normal division does not follow, and many cells then autolyze. He observed these changes in bacteria at dilutions of penicillin well above those which were completely inhibitory to their growth. For this reason he felt that some action might be expected of penicillin *in vivo* at concentrations much lower than those needed for complete inhibition.

Since this interesting observation of Gardner, many others have noted similar morphologic changes in bacteria. Fleming (1941), in a discussion of the bacteriostatic effect of penicillin, referred to the fact that in low dilutions it affects the morphology of bacteria and interferes with their division. Smith and Hay (1942) noted a marked increase in size of staphylococci exposed to inhibiting concentrations of penicillin and found that the cells then underwent lysis, leaving behind a granular material in the sediment. This swelling and lysis were apparently only associated with the *active growth* of the staphylococci, suggesting to the authors that penicillin either acts directly on the cellular wall or prevents the assimilation of certain growth factors necessary for actual fission of the growing cell. *Fully grown* suspensions of the organisms did not show these changes, even when high concentrations of penicillin were used. Weiss (1943) noted similar changes in size and shape of staphylococci, streptococci, and *Clostridium welchii* and demonstrated these in electron micrographs. Dubos (1944), in a review of the action of various antimicrobial agents, classifies the action of penicillin as bacteriostatic and feels that its production of giant forms in the inhibited cells is evidence that it may affect some step of cellular division rather than "a catabolic process measured by respiration." The studies of Miller *et al.* (1944) on gonococcal urethritis demonstrate the fact that the same type of reaction seems to take place *in vivo*, as greatly enlarged gonococci were found in phagocytes from urethral smears as early as 2 hours after intramuscular injection of penicillin. The meningococcus was found to be similarly affected *in vitro* (Miller and Foster, 1944). The observations of many workers support the conclusion that young, rapidly growing organisms are the ones which are the most susceptible to the action of penicillin, and that substances in the media which enhance the growth of the bacteria seem to render them more sensitive to its action (Hobby *et al.*, 1942; Bigger, 1944; Hobby and Dawson, 1944; Lee *et al.*, 1944; Miller and Foster, 1944; Rantz and Kirby, 1944). As a general rule the corollary to this is also true, for if the bacteria are inoculated into a medium which is lacking in some of the growth requirements, the efficiency of the penicillin is definitely lowered. An exception to this general rule has been pointed out by Garrod (1945), for he found that penicillin was more active at a temperature of 42 C—although growth of the bacteria had stopped—than it was at 37 C.

Most of these authors likewise noted the occurrence of lysis in their cultures as a further step in the action of penicillin. This was particularly true in the case of the staphylococcus, though it was found to occur in other organisms also. Todd (1945) observed bacteriolysis in all the penicillin-sensitive organisms tested, including the pneumococcus, viridans streptococcus, hemolytic streptococcus, staphylococcus, and *Clostridium welchii.* The most rapid lysis occurred in organisms at the maximal rate of multiplication.

A report by Chain and Duthie (1945) is of particular interest in this regard. They studied the oxygen uptake of cultures of staphylococci in the presence of penicillin in various phases of growth and found the organisms to be most susceptible during the lag and logarithmic phases of active growth, but not affected even by high concentrations of penicillin in the "resting" phase. It is their opinion that penicillin "appears to interfere with a metabolic function involved in the early stages of bacterial growth."

Our own studies were concerned, first, with observations on the effect of penicillin on the morphology of the staphylococcus and streptococcus *in vitro* and, secondly, with its action on the pneumococcus and its capsule, both *in vitro* and *in vivo.*

STAPHYLOCOCCUS EXPERIMENTS

Methods. Penicillin-sensitive staphylococci were grown in beef infusion broth and tested with penicillin at various periods of their growth, also during the lag phase and after their multiplication had stopped. The penicillin employed in these first experiments was the crude form, prepared from two different strains of *Penicillium,* the usual dilutions in the cultures being approximately 0.5 to 0.125 units per ml. Heavy inocula of staphylococci were used, 0.1 ml of the whole broth culture, in order to ensure adequate organisms for the microscopic and photographic studies. Representative specimens of the penicillin-treated cultures and the controls were spun down in a centrifuge at various time intervals, and a sample of the sediment was spread on a slide and fixed with heat. The films were then stained on a single slide in a very dilute solution of carbol

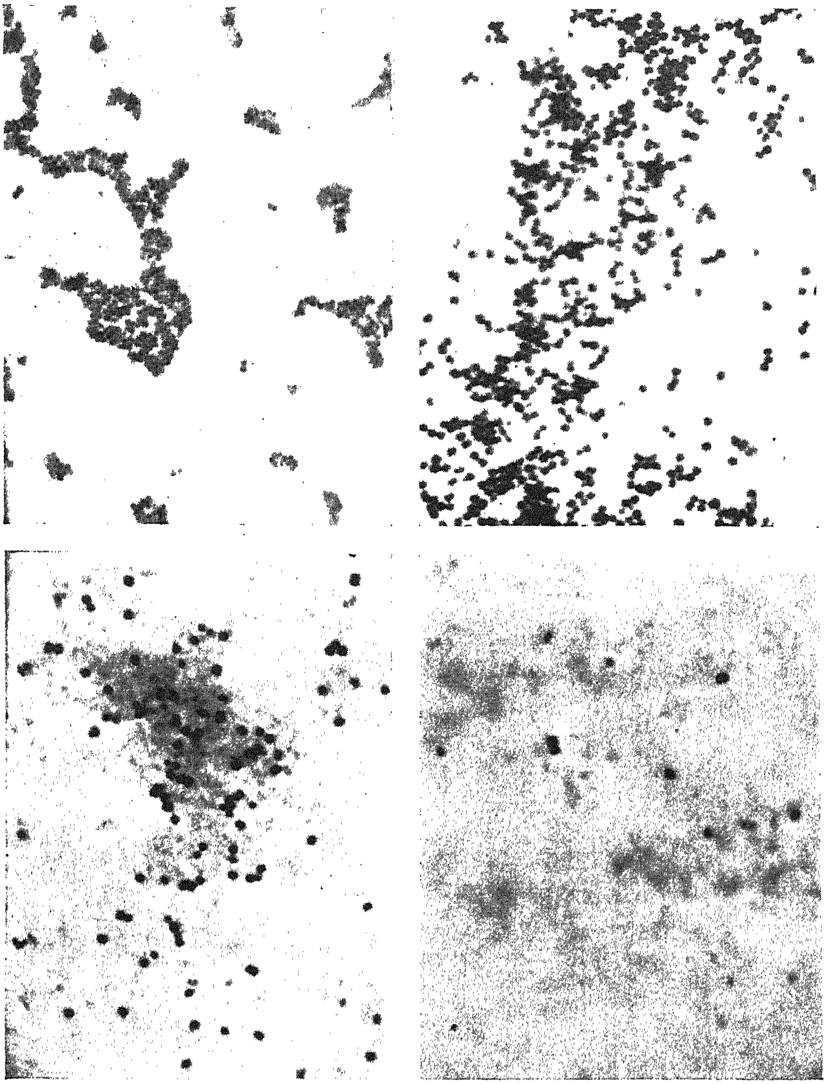

FIG. 1. STAPHYLOCOCCUS SERIES
Mag. × 1,300

Upper left: Control staphylococcus culture at 3 hours.

Upper right: Staphylococcus with penicillin at 3 hours. The organisms are all enlarged and deeply stained.

Lower left: Staphylococcus with penicillin at 7½ hours. Abnormal staphylococci and much granular debris.

Lower right: Same at 10 hours. Very few and markedly altered bacteria are left. Debris of lysed cells. Culture sterile at this stage.

fuchsin for about 30 minutes, and photomicrographs were made from these preparations.[1] The results are illustrated in figure 1.

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[1] All the photographs were taken by Mr. Joseph Drane and Mr. John McQuaid in the Department of Anatomy.

It is clearly shown in these pictures that the first effect of penicillin on the staphylococcus which is observable microscopically is the production of marked enlargement of the cell body, with a definite increase in the intensity of its staining properties by dilute carbol fuchsin. This is followed by a dissolution of the bacterial cell, which is quite marked in 7½ hours, and complete, involving practically all the organisms, in 10 hours. The staphylococcus from the control tube at 10 hours had exactly the same appearance as at 3 hours. Subcultures of the centrifuged sediment taken at these same intervals revealed a marked decrease in the number of surviving organisms at each stage, with sterility in 10 hours. Similar results were obtained with several other strains of staphylococci.

This same phenomenon was observed if the inoculum of bacteria was taken very early in the growth period, i.e., at one hour's growth, presumably during the lag phase. Similar results were obtained if the cultures were taken at 2½ hours, i.e., in the early logarithmic phase of growth. On the other hand, no such morphologic changes resulted when penicillin was added to a tube containing an old, fully grown culture of a penicillin-sensitive staphylococcus, this culture having been in the incubator for 10 days previously. Likewise, when penicillin was added to a culture of a sensitive strain in the usual way and then placed at once in the icebox for 18 hours instead of the incubator, no recognizable changes in shape and no evidence of bacteriolysis were observed, and subcultures remained strongly positive. Two strains of penicillin-resistant staphylococci were treated in a similar manner with no change in their morphology.

These findings are in agreement with the conclusions of other workers and show that the action of penicillin on the staphylococcus, as observed microscopically, is one which leads at first to enlargement of the bacterial cell and then to its dissolution. With even higher dilutions of penicillin, though the staphylococci are not killed in the foregoing manner, the appearance of many of them is altered in such a way as to suggest that they represent degenerate forms which would presumably be more easily destroyed by the phagocytes of the body. This evidence of penicillin activity at very high dilutions may help explain the fact that it seems to influence infections at times when it is present in the blood in such small quantities as to be undetectable by the standard methods for its determination.

STREPTOCOCCUS EXPERIMENTS

Methods. These studies in general were similar to those employed for the staphylococci. Heavy inocula of 18-hour cultures of group A beta hemolytic streptococci were added to broth enriched with a small amount of rabbit's blood, and crude penicillin was added to this preparation in final dilutions of approximately 0.5 to 0.25 units per ml. These preparations were then spun down, slowly at first to get rid of most of the blood cells, then rapidly for 15 minutes. Films and cultures were made from the sediment and these films were all stained on the same slide with a dilute solution of carbol fuchsin for approximately 30 minutes. Photomicrographs were then made from these stained films. These results are shown in figure 2.

The photographs demonstrate the marked inhibition in growth of this culture of group A beta hemolytic streptococcus, this being corroborated by the cultures which became nearly sterile at 10 hours and completely so at 24 hours. They also show the formation of the "giant forms" which have been remarked on by others, and there is no evidence of debris to suggest that many of the bacteria have become lysed.

It seems, from this experiment at least, that the group A streptococcus is not killed in the same manner as the staphylococcus by penicillin. Only a moderate number of enlarged forms are to be found, and though these probably do become broken up, the majority of the organisms apparently do not undergo complete dissolution. This is in agreement with findings reported by Hobby *et al.* (1942), these authors having found that penicillin did not cause lysis of streptococci, though this reaction was observed in some later experiments (Hobby and Dawson, 1944).

PNEUMOCOCCUS EXPERIMENTS

In the studies of the effect of penicillin on the pneumococcus we were interested in two reactions, (1) the effect on the organism itself and (2) the effect on its capsule.

In Vitro Tests

Methods. The medium employed was beef infusion broth enriched either with human serum, rabbit's blood, or ascitic fluid. Crude or purified penicillin was added to this in amounts usually approximating 1 to 0.1 unit per ml, and heavy inocula of 18-hour cultures of pneumococci (0.3 to 0.5 ml) were added to this, and then placed in the incubator. Cultures and microscopic preparations were then made at various intervals after centrifuging. The bacterial stains employed in these experiments were the gram stain and the dilute carbol fuchsin. The capsule stain was a modification of that described by Butt *et al.* (1936). These authors recommended mixing a loopful of the material to be stained with an equal amount of 6 per cent glucose solution, then with india ink. This was spread on a microscopic slide, fixed with methyl alcohol, and counterstained with methylene blue. A counterstain of safranine proved much more satisfactory in our hands than methylene blue. This was at first applied for a few minutes, but stains of longer duration gave much better definition of the bacteria in the capsules; the best preparation resulting from a stain of 1 to 2 hours. It is, of course, absolutely necessary that the control and penicillin-treated specimens be prepared in exactly the same manner and stained for an equal length of time. The results of these *in vitro* studies are shown in figures 3 and 4.

The following observations were made on the gross and microscopic appearance of the pneumococcus cultures. In a typical experiment penicillin dilutions of 5, 1, 0.1, and 0.01 units per ml and a control culture were used. The first three of these tubes remained clear throughout; they were nearly sterile at 6 hours and completely so at 18 hours. The tube containing 0.01 unit penicillin per ml was almost as cloudy as the control at 6 hours, but completely clear and nearly sterile at 18 hours. This indicated slight inhibition at this highest dilution in

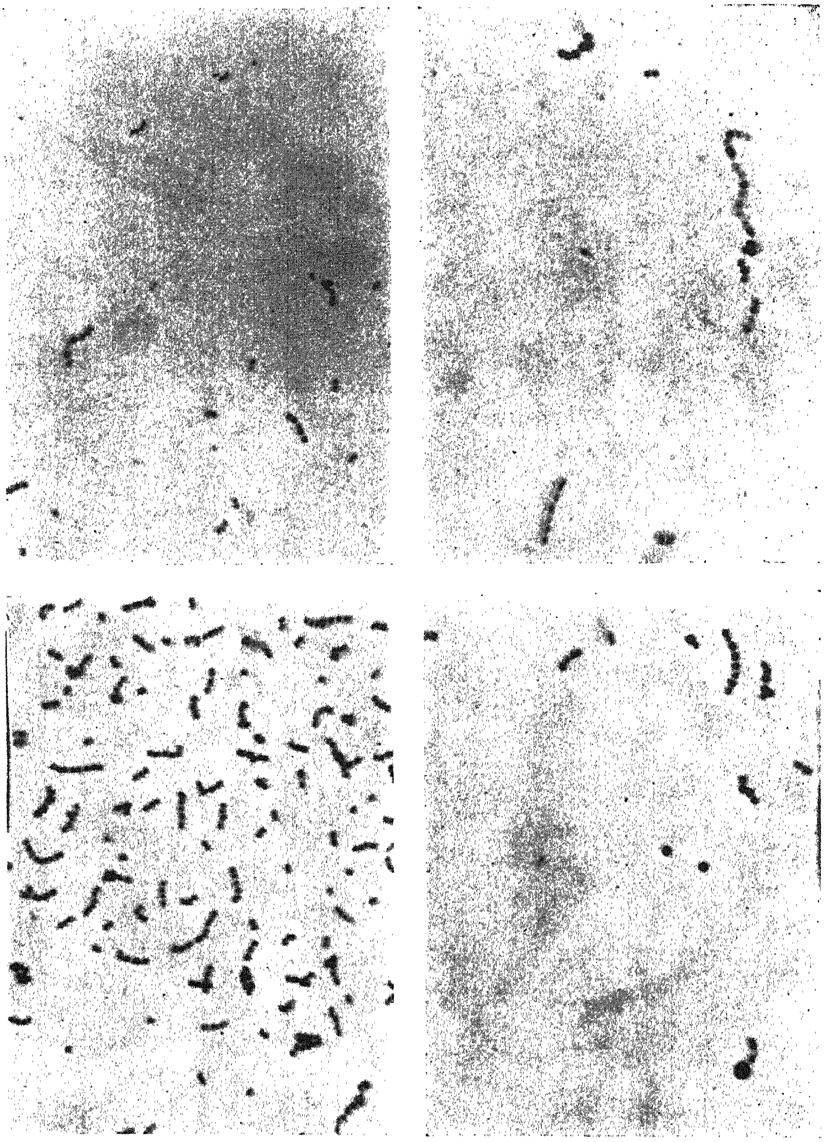

FIG. 2. STREPTOCOCCUS SERIES

Mag. × 1,300

Upper left: Streptococcus control at 3 hours.

Upper right: Streptococcus with penicillin at 3 hours. Early changes in morphology and staining may be noted.

Lower left: Control at 7½ hours.

Lower right: Streptococcus with penicillin at 7½ hours. Note the appearance of giant forms and marked reduction in number of organisms.

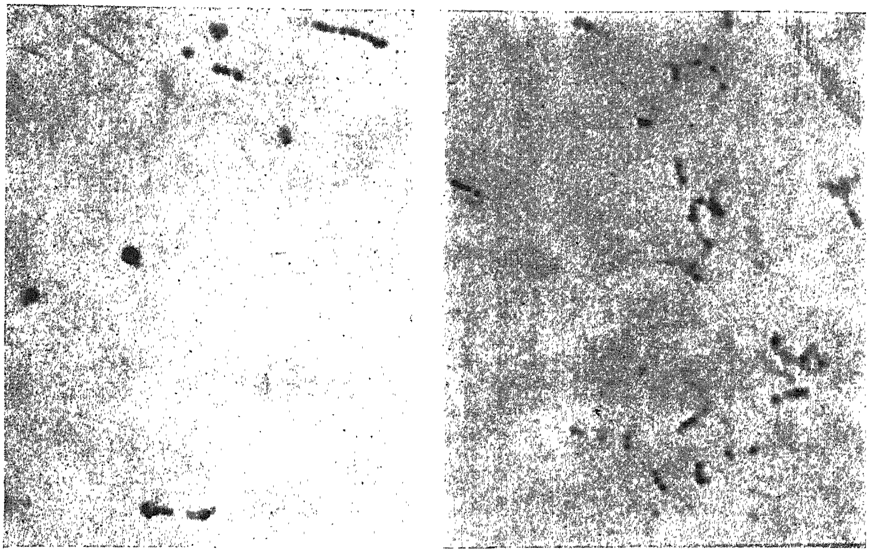

FIG. 2—Continued

Left: Penicillin-streptococcus preparation at 10 hours.

Right: Same at 24 hours. Most of the bacteria appear dead. Culture sterile at this point. Note persistence of chain forms and visible bacterial cells.

the early stages, followed by lysis. Thus the penicillin could be said either to cause lysis of the pneumococci or to accelerate the natural tendency of these organisms to autolyze. Gram stains of the centrifuged sediment of the first three tubes showed a few organisms almost all of which were gram-negative, usually occurring singly, a few diplococci, and almost no chains. Stains from the tube showing slight inhibition showed a picture very much like the control, except that most of the bacteria were gram-negative. There were chains just as in the control and only an occasional form that seemed somewhat larger than normal.

As for the studies on the pneumococcus capsule, in these experiments and many similar ones it was established that by 3 hours there were both a marked reduction in the total number of capsules to be found in a preparation and a consistent tendency for the majority of them to appear entirely *empty* or to contain a small amount of irregularly shaped bacterial substance. The capsules themselves were not perceptibly altered. Similar but less advanced changes were noted by 2 hours. Though no very high dilutions of penicillin were employed, it was found that, when its strength was 0.1 or 0.5 unit per ml, this capsule phenomenon was more easily demonstrated than when 1 unit per ml was used. In the latter case the most striking finding was a great reduction in the number of organisms—even in 3 hours both by culture and by film—making it difficult to find more than a few scattered capsules, though very heavy inocula had been employed.

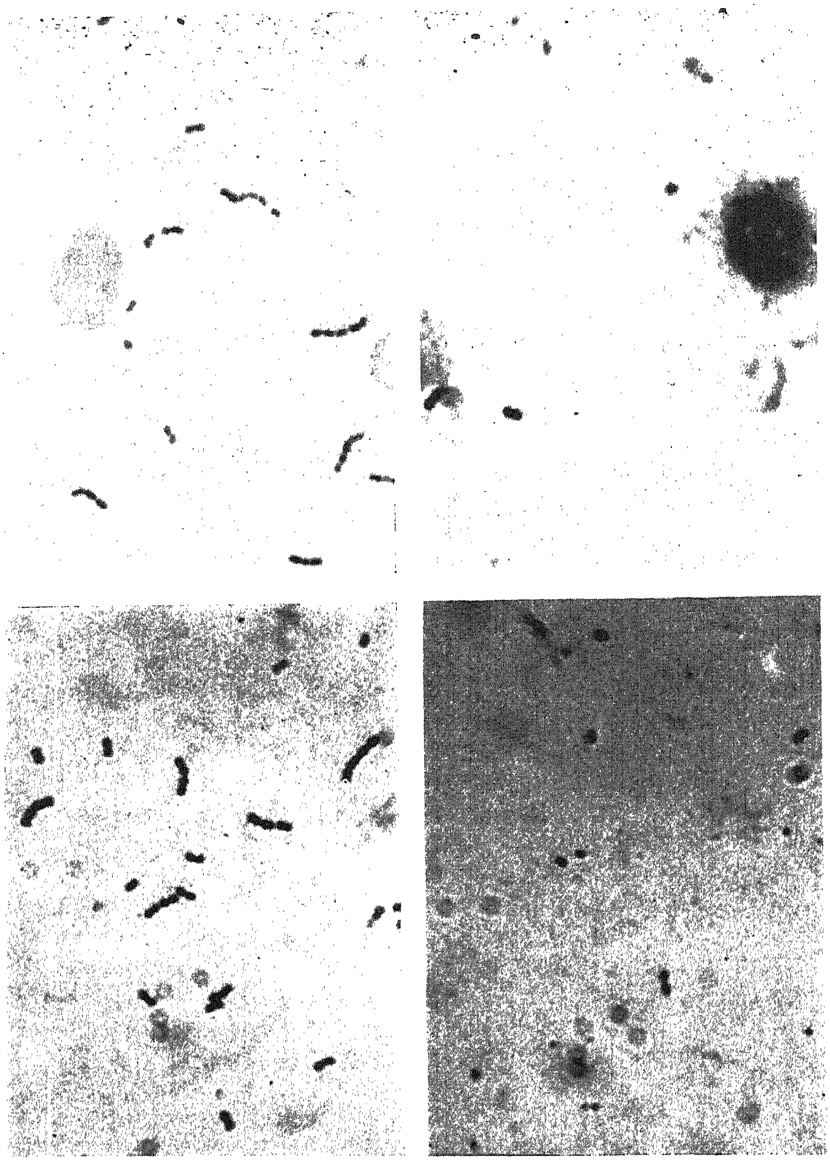

FIG. 3. PNEUMOCOCCUS SERIES

Mag. × 1,250

Upper left: Pneumococcus type I control at 3 hours. Note tendency to formation of short chains. (Carbol fuchsin stain.)

Upper right: Pneumococcus type I with penicillin. One unit per ml at 3 hours. Organisms reduced in number and deeply stained.

Lower left: Pneumococcus type III control at 6½ hours. (Gram stain.)

Lower right: Pneumococcus type III with penicillin 0.1 unit per ml at 6½ hours.

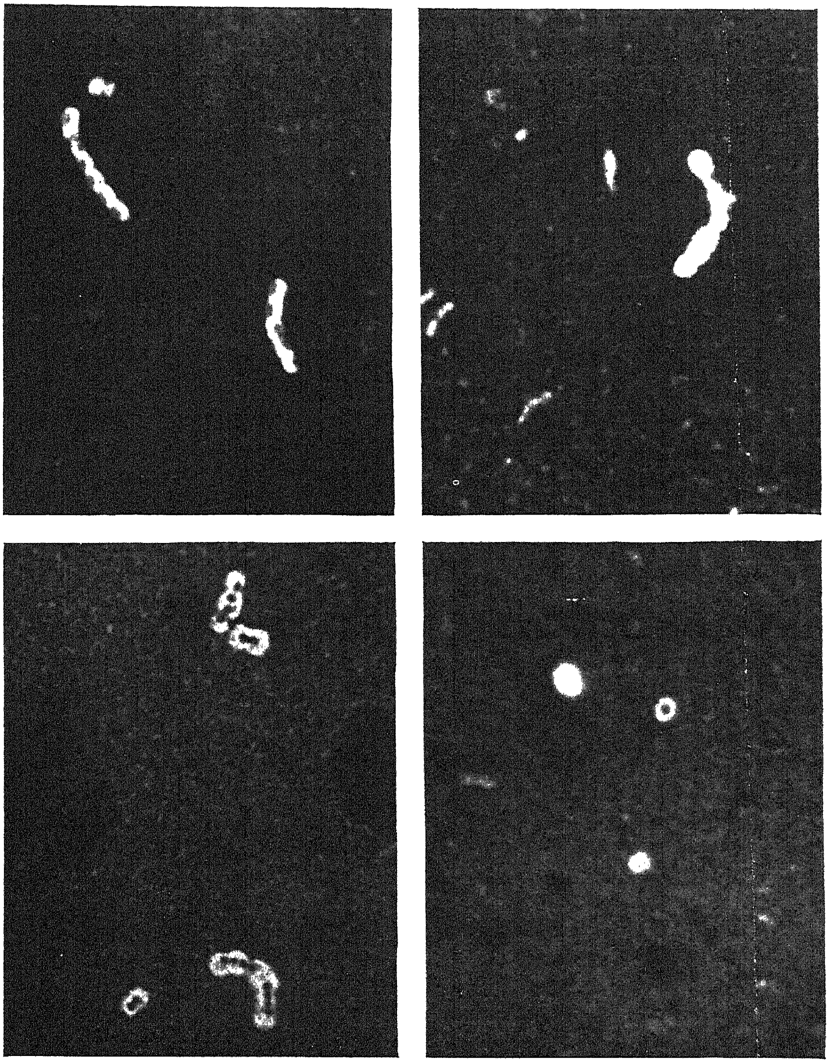

FIG. 4. PNEUMOCOCCUS CAPSULE SERIES

Upper left: Pneumococcus type I control at 3 hours. (Same strain with chain formation as shown in figure 3, upper left.)

Upper right: Pneumococcus type I with penicillin. 1 unit per ml, at 3 hours. Note almost complete absence of stained bacteria in the single capsule. (Other organisms in the background are contaminants in the india ink.)

Lower left: Pneumococcus type III control at 6½ hours.

Lower right: Pneumococcus type III with penicillin 0.1 unit per ml at 6½ hours.

In Vivo Tests

Mouse experiments. It next seemed of interest to determine whether or not this capsular phenomenon could be reproduced *in vivo*. Mice were used in these tests, and cultures of a type III pneumococcus and penicillin were injected intraperitoneally. Two types of inocula were employed, one being a heavy suspension of the pneumococci obtained in saline from the peritoneal cavity of mice injected with a culture of pneumococci a few hours previously and the other an 18-hour culture in blood broth or serum broth. Heavy inocula were used of approximately 1 ml, which were injected intraperitoneally and followed in about 2 hours by intraperitoneal injections of crude or purified penicillin in strengths of 100 to 250 units. The mice were killed at intervals of 4 to 8 hours, and the contents of the peritoneal cavities were washed out with approximately 1 ml of normal saline or broth. This was centrifuged, slowly at first to throw down most of the cellular elements and then rapidly to obtain the bacteria. The sediment was washed again with saline or broth in order to eliminate most of the serum—as this causes clumping of the india ink particles, thus resulting in an unsatisfactory background for the capsule stain. Preparations with india ink were then made from these sediments, fixed, and stained in the usual way. Control preparations were made for each test and treated in exactly the same way as those in which penicillin was used.

The results of two typical experiments are demonstrated in figure 5. In these the capsules are very large, no doubt because a type III pneumococcus was used and grown in the mouse's peritoneum. The findings were essentially the same whether crude or purified penicillin was used. These and many other similar experiments made it clear that the phenomena noted *in vitro* could be reproduced *in vivo.* The capsules seem to be normal in structure and the pneumococci appear to have been either completely destroyed, leaving no trace behind or leaving remnants that stain very faintly. These remnants at times appeared as definite small diplococci, and at other times there was only a small amount of granular material to be seen. A few normal-looking pneumococci could nearly always be found in the same preparation. Subcultures taken at the time these preparations were made invariably showed a marked reduction in the number of viable organisms present, and an occasional culture would be sterile.

Human experiments. A good many observations on patients with pneumococcus infections under treatment with penicillin indicate that this "empty-capsule" phenomenon is a very common, if not a regular, occurrence under such circumstances. Miss Kathleen Foley and others in the bacteriological laboratory noticed, on setting up a *quellung* preparation on some pus from an empyema which had been treated locally with penicillin, that capsules were present which "quelled" normally but that either no pneumococci or only very faintly stained ones were visible within the capsules. We confirmed this finding on several other cases under similar treatment.

It was found that the same reaction could be demonstrated in the sputum of patients with lobar pneumonia. The characteristics of the sputum made it difficult to obtain good preparations with the india ink. This could be done, however, by shaking the specimen up with sterile broth, filtering through sterile filter paper, and then centrifuging. Preparations were then made from the

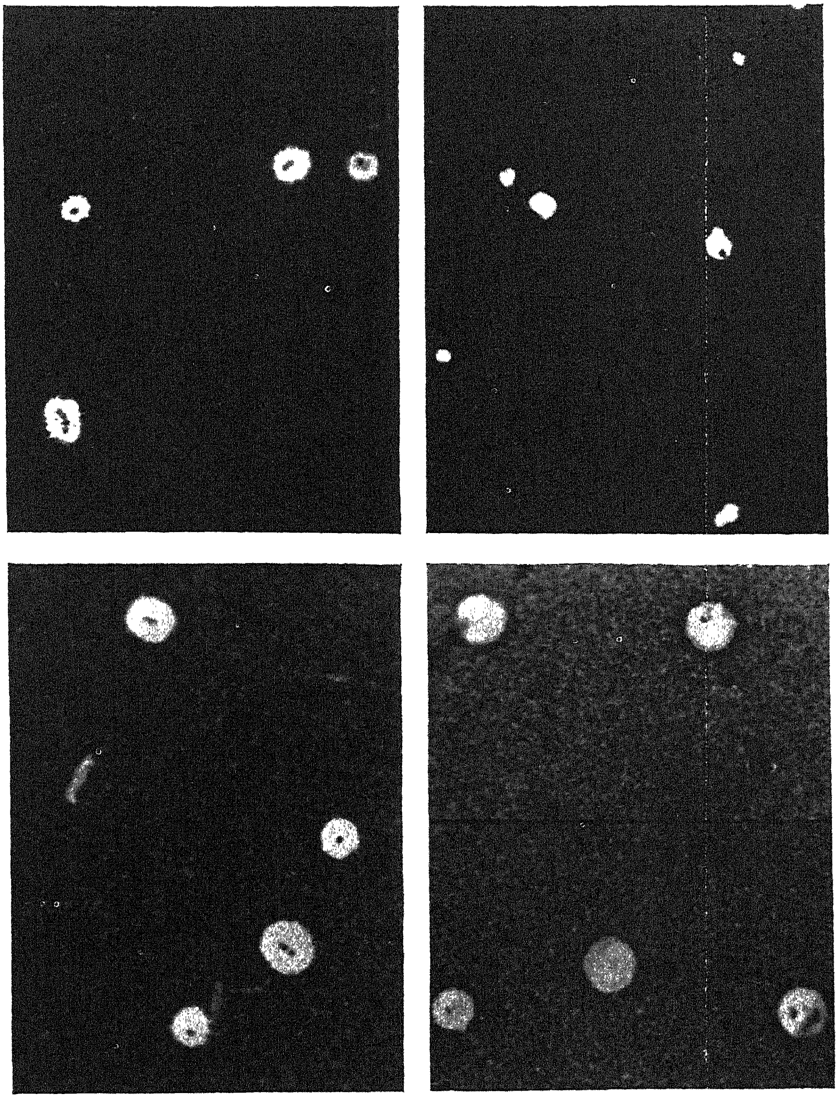

FIG. 5. PNEUMOCOCCUS SERIES, MOUSE EXPERIMENTS
Mag. × 1,200

Upper left: Pneumococcus type III control. From mouse peritoneum after 1½ hours.
Upper right: Pneumococcus type III with sodium penicillin, 100 units. After 4½ hours in mouse peritoneum. (Capsules appear smaller because of very dense background.)
Lower left: Pneumococcus type III control from mouse after 4 hours.
Lower right: Pneumococcus type III from mouse after 4 hours. Top—With crude penicillin, 100 units. Bottom—Same with sodium penicillin, 250 units.

sediment and stained for capsules. Cultures from the sediment before penicillin therapy were nearly pure for pneumococci and after treatment were often nearly sterile. In three cases satisfactory preparations were obtained in this way before, and the day following, institution of intramuscular therapy with penicillin in doses of 30,000 units every 3 hours. Most of the capsules which were found after therapy appeared empty, whereas those before treatment showed pneumococci which stained normally.

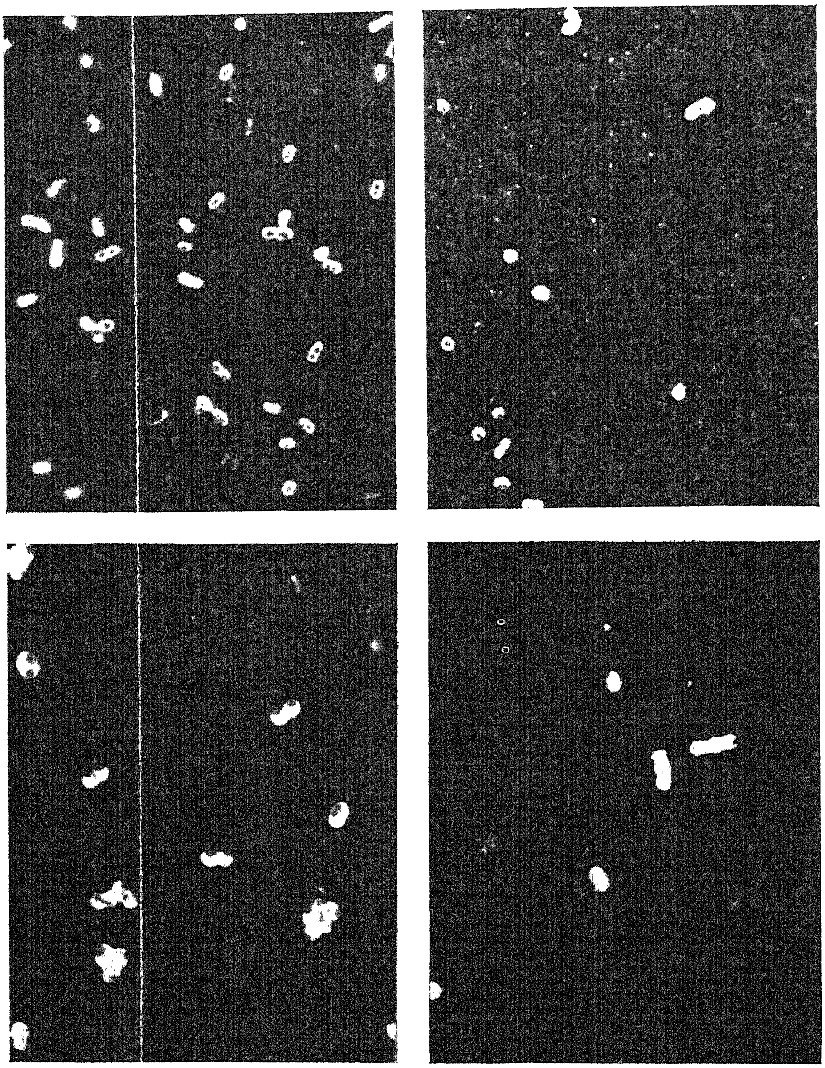

FIG. 6. PNEUMOCOCCUS SERIES, HUMAN INFECTIONS
Mag. × 1,200
Upper left: Pneumococcus type XII meningitis before penicillin therapy.
Upper right: Same 4 hours after 20,000 units penicillin intrathecally.
Lower left: Pneumococcus type II meningitis before penicillin therapy.
Lower right: Same 20 hours after 20,000 units penicillin intrathecally.

It was interesting to note also that two of these patients had been taking one of the sulfonamides at the time of admission and just before the control preparation was made. This suggests that the pneumococcus does not exhibit this type of reaction when exposed to sulfonamides. *In vitro* experiments performed later with strongly and weakly inhibiting concentrations of sulfadiazine and sulfamerazine bore this out, as the pneumococci under such circumstances were found to stain normally in their capsules.

Material was obtained from two patients with pneumococcus meningitis. The results of the studies on their spinal fluids are illustrated in the photographs in figure 6. The first patient had an overwhelming bacteremia and meningitis due to a type XII pneumococcus and died 4 hours after the diagnosis was made. The second specimen in this case was taken by spinal puncture immediately after death—approximately 4 hours after 20,000 units of penicillin had been injected intraspinally. In this preparation it was found by actual count that 50 per cent of the capsules appeared to be empty, whereas only one in five had this appearance before the therapy. The other patient had a type II pneumococcus in the spinal fluid and was given 20,000 units intraspinally. The second sample of spinal fluid was taken 20 hours after this injection, and nearly all the capsules were empty or showed pneumococci which stained very faintly. The bacteriologist doing the routine *quellung* on this material remarked on the peculiar microscopic appearance of the preparation, for the organisms themselves had failed to take the methylene blue stain, and many capsules were left which in some ways resembled red blood cells.

DISCUSSION

It seems clear from this study that though penicillin may have one fundamental effect on bacterial metabolism which holds true for all susceptible organisms, it has varying effects on the cellular structure of the bacteria, depending on the type of organism under consideration. This holds true at least for the three types of bacteria dealt with in this investigation. It is also clear from this, and many other observations, that the bacteria are actually *killed* by penicillin and without the aid of the defensive mechanisms of the body, rather than simply being *inhibited* in their growth. This bactericidal action does not take place immediately—as it does with many antibacterial agents—but subcultures show that during the first 3 hours great numbers of bacteria die if exposed to penicillin in sufficient concentration, and by 10 hours the cultures are usually sterile. These cultural changes may be correlated with actual alterations in the morphology of the various bacteria, as demonstrated by the microscopic studies illustrated in this report.

Though the degree and type of the structural changes in the different bacteria vary considerably, one effect of penicillin on the bacterial cell body results in enlargement of the cell. This reaction is most marked at the higher effective dilutions of penicillin. It is interesting to compare this phase of the reaction with the lag period of normal bacterial growth. This period corresponds roughly to the first 1½ hours of life of a fresh culture, and during this time there is little or no evidence of multiplication of the bacteria. They are not in a resting stage, however, but show normal metabolic activity, which results in *growth* and actual enlargement of bacterial cells (Topley and Wilson, 1938). The normal stimulus to division then takes place, and the bacteria are started on the phase of "active growth." The reasoning which has been advanced that penicillin interferes with this normal stimulus to division seems to offer the most logical explanation for this primary inhibiting effect of the drug. It seems to produce at first an exaggerated lag phase, which in our experiments was much more marked for the staphylococcus than for the streptococcus, and least for the pneumococcus.

In the case of the staphylococcus this phase in which there is inhibition of division and swelling of the bacteria is followed by dissolution of the cells until little remains of the culture but granular debris. These morphologic changes in the staphylococci were noted only in the case of the growing organisms; they were not produced at icebox temperature nor in the old cultures in the resting phase. Resistant strains did not show any such structural alteration when exposed to penicillin. Sulfathiazole failed to induce this reaction when tested with staphylococci either in weakly or strongly inhibiting concentrations.

Definite evidence of complete dissolution of cellular structure was not observed in the cultures of streptococci; and though a good many giant forms were seen during the phase of inhibition—these presumably becoming lysed—the majority of the organisms did not exhibit these alterations, and some chains were still apparent though the cultures became sterile.

It may be mentioned here that sulfonamides have also been found capable of changing the morphology of streptococci, for Gay and Clark (1937), Lockwood (1938), and Tunnicliff (1939) noted enlargement of the individual bacteria and elongation of their chains when exposed to sulfanilamide. Such organisms were found to be more susceptible to phagocytosis than were the controls (Tunnicliff, 1939).

The effect of penicillin on the morphology of the pneumococcus as shown by the stain employed in this work was one of enlargement of a few of the cells, but usually, except for a marked diminution in the number of bacteria, there would be little change from the control preparation. Most of the remaining bacteria, however, did become gram-negative. The changes noted relative to the *capsules* were unexpected and very interesting. The capsule being intimately associated not only with the type specificity but with the virulence of this species, we suspected that it would have to be destroyed before the pneumococcus could be killed by the penicillin. It was interesting, therefore, to find that after the organism itself had evidently been killed and failed to take the safranine stain, the capsular substance remained intact. In this condition it was still capable of reacting in the usual way when exposed to type-specific serum. It appears that the penicillin either passes through the capsule by diffusion and then destroys the organism, or is actively absorbed by the growing pneumococcus and passed through its capsule during the process of bacterial metabolism. That the latter explanation seems the more likely is indicated by the fact that the phenomenon is not observed at icebox temperature; which suggests that it occurs only when the experiments are performed under conditions favorable to active growth of the bacteria. This is not merely an *in vitro* phenomenon, for it was observed in mice and in several types of pneumococcal infections in human beings. The fact that it was found in the sputum of patients with lobar pneumonia during penicillin therapy indicates that it may take place in high dilutions of the antibiotic. This may be a reaction which is unique for penicillin; at least there has been found no evidence for its occurrence with sulfonamides either *in vitro* or in patients with pneumonia.

It is thus shown that the pneumococcus capsule is easily penetrated by penicillin and does not offer the organism any protection against it. It seems reasonable to presume that the way in which the bacterial cell itself is destroyed in this reaction is by the penicillin's producing enough injury to promote an acceleration of the pneumococcus' natural tendency to autolysis.

Another feature worth emphasizing again for its clinical significance is the production by penicillin of slight inhibiting and morphologic changes in bacteria at higher dilutions than those which are observed in the gross *in vitro* tests of an organism's penicillin sensitivity. Bacteria which are only slightly affected in such a manner are almost certainly altered enough to render them more susceptible to the normal immune mechanisms of the host. Such observations may help explain the finding that penicillin is frequently effective in bacterial infections despite the fact that it is present in the blood and tissue fluids in too low a concentration to enable its detection by the routine methods that are now employed.

All the observations reported in this study support the general belief, expressed by many workers in this field, that penicillin is most effective against young, rapidly growing bacteria. This is one of the features of its action that is of the greatest interest and importance from the clinical standpoint. The fact that penicillin apparently does not affect the bacteria in the resting stage—as shown by its failure to produce morphologic or cultural changes in very old cultures or at icebox temperature—proves that this is not merely a physical or chemical effect on the body of the cell, but one that influences some phase of the organism's metabolic and reproductive activity.

SUMMARY

The *in vitro* action of penicillin on staphylococci causes enlargement of the bacterial cells followed by lysis. This is apparently due to interference with their normal stimulus to division and multiplication rather than to complete inhibition of growth.

The same effect is apparent to a lesser degree on cultures of beta hemolytic streptococci and pneumococci. In the case of the pneumococcus at least, the capsule remains intact for some time after the cell body is destroyed.

This effect on the pneumococcus is apparently a biologic rather than a physical or chemical reaction, since it occurs only under conditions in which the organism is capable of growth.

These various morphologic changes are noted to some extent in the staph-

ylococci within the first 2 hours of their exposure to penicillin, and in the streptococci and pneumococci in 3 hours.

The clinical significance of these findings is discussed.

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CITRIC ACID FERMENTATION OF BEET MOLASSES[1]

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Beet molasses constitutes an economical source of carbohydrate for industrial processes. Successful utilization of it in citric acid fermentation by molds is highly desirable. Although beet molasses presumably is now being used industrially, such commercial methods are closely guarded trade secrets. Moreover, its use may be limited. Thus, von Loesecke (1945) states, "It is believed that . . . in present commercial practice it is necessary to use beet molasses from a certain locality to achieve efficient operation." The literature concerning the fermentation apparently contains no fully satisfactory procedure for utilization of beet molasses in existing plant conditions, although applicable studies have been reported. The ultimate object of this research, therefore, was to develop a method by which beet molasses may be improved for use as a substrate in mycological citric acid production, giving yields comparable to those obtained with synthetic media.

The literature has been adequately reviewed by Wells and Herrick (1938), Prescott and Dunn (1940), and von Loesecke (1945). Relatively few studies have been concerned with beet molasses or other unrefined sugar sources. Apparently the only specific reference to the use of beet molasses was reported by Roberts and Murphy (1944); they developed a method in which a beet molasses medium, impregnated on sphagnum moss, was fermented by a strain of *Aspergillus niger* to yield 25 to 30 g of calcium citrate per 200 g of molasses in 2 days. Previously, Cahn (1935) had reported a similar process, using cane molasses. Chatterjee (1942), Palei and Frantzuzova (1936), Bernhauer, Iglauer, and Knobloch (1941), Das Gupta, Saha, and Guha (1940) have also reported studies dealing with the fermentation of various molasses or raw sugars.

METHODS

Three types of containers were employed. Preliminary experiments were conducted in 6-oz bottles, the bottle being laid on the side to provide a large surface area with approximately 1-cm depth of medium. This furnished maximum contact and utilization of the nutrients of the substrate by the mold mycelium; the fermentation would thus be expected to respond more markedly to experimental modification in the substrate. Smooth-walled glass tumblers were used subsequently to simulate shallow pan conditions. In some cases, plant conditions were more closely duplicated by the use of shallow aluminum pans 36 x 25.5 x 7.5 cm in size, containing 5 liters of medium to give a depth of 5 cm. The bottles were plugged with cotton, the tumblers were covered with "airmat," and the pans were loosely covered with a piece of sheet aluminum.

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The mold used was *Aspergillus niger*. Cultures were stored as soil suspensions, according to the recommendations of Greene and Fred (1934). Seed cultures for the fermentation were prepared by inoculation from the soil suspension to bottle plates of sucrose nutrient agar. The plates thus inoculated were incubated for 6 to 25 days. A standard suspension of spores was prepared by washing off these cultures with distilled water to approximate 50,000,000 spores per ml. In the case of bottles or tumblers, 1 ml of the spore suspension was used as inoculum; for the aluminum pans, 20 ml were used The containers were then gently swirled or agitated to ensure an even distribution of spores. The inoculated containers were incubated at 30 C; analyses were usually made at 8, 10, and 12 days. Unless otherwise specified, the results given are for a 10-day fermentation period. The loss in volume at this time was measured directly by determining the difference between the volume of the fermented liquor, including the mycelium mat, and the original volume; this loss was expressed as percentage of loss in volume. The fermented liquor and mat were then diluted and steamed for 15 minutes, in order to kill the organism and to extract residual acid from the mat. After cooling, the liquor and mat were diluted to a given volume, thoroughly mixed, and aliquots taken for analyses.

Residual sugar in the fermented liquor, as well as sugar in the original medium, was determined by the method of Somogyi (1937).

Total acidity was determined by titration with 0.1 N sodium hydroxide, to the phenophthalein end point. Yields were calculated as the percentage of conversion of the sugar supplied to citric acid monohydrate, assuming it to be the only acid product present. By this method, a theoretical yield of 123 per cent would be possible, if all the sugar carbon were converted to citric acid carbon. Although this representation of yields was not necessarily accurate, it nonetheless permitted rapid gross differentiation of results.

Under the conditions used, citric and oxalic acid made up virtually all of the acid produced by the fermentation. Thus, it was possible to determine the amount of oxalic acid produced, and then merely to calculate, by difference, the amount of citric acid produced This procedure was carried out when differential evaluations of citric and oxalic acid yields were desired. An oxalic acid microanalysis was standardized for accurate use with molasses media; the method consisted of the usual precipitation with calcium chloride, centrifuging, decanting, and titrating the calcium oxalate while hot with 0.1 N ceric sulfate solution.

EXPERIMENTAL

Strains. In the preliminary work a strain of *Aspergillus niger* was selected which would produce a maximum amount of citric acid in the shortest time and be adapted to fermentation of a beet molasses substrate. A comparison of 20 strains contained in our culture collection showed two strains to be markedly superior in their fermentative ability. Subsequent work was largely restricted to use of these two strains. Strain 62 is the culture no. 67 employed by Wells, Moyer, and May (1936); no. 72 was presumably *A. niger* 1015 of the American Type Culture Collection. Although no. 62 was superior on synthetic medium, it gave inferior results on beet molasses medium (see table 6). On the other hand, strain 72, although inferior on synthetic medium, proved to be superior on beet molasses media and subsequently was used routinely in these studies. Although no. 72 formed relatively large proportions of oxalic acid, it nonetheless gave the highest citric acid yields and its deeply invaginated mycelium mat made it adaptable to deep layers of medium. In general, strain 72 could be expected to produce citric acid yields of approximately 50 per cent with properly prepared beet molasses medium over a 10-day fermentation period (see table 6).

TABLE 1

Variation in fermentative ability of single-spore-derived cultures of A. niger no. 62 on synthetic medium

STRAIN NO.	PERCENTAGE YIELDS, TOTAL ACIDITY			
	7 days	8 days	9 days	10 days
62	42.5	44.0	52.2	46.7
103	49.1	51.1	51.1	50.4
105	63.4	58.9	63.3	61.4
106	52.6	57.6	59.1	59.3
107	51.8	60.8	61.5	62.1
108	57.2	58.7	58.4	58.7
111	48.9	48.3	47.4	40.9
113	58.4	61.9	61.4	62.3
116	56.9	61.9	60.4	61.0
117	55.2	61.7	63.4	58.2
120	48.9	54.6	56.0	55.7
124	55.2	56.6	53.2	53.3
131	56.3	53.1	53.1	53.5
132	44.2	42.2	42.6	37.1
135	57.6	54.2	55.8	60.0
143	52.8	62.1	65.8	63.6
156	49.1	47.4	47.6	52.8
158	47.6	55.2	55.0	59.7
160	52.8	55.8	63.2	64.6
161	53.2	62.4	60.0	58.0
165	54.2	61.6	58.5	60.8

No newly isolated strains of *Aspergillus niger* from natural sources gave yields consistently better than no. 62 or no. 72. In some instances, an apparently superior strain was isolated, but on continued cultivation the property was rapidly lost.

It was hoped that an existing strain might be improved in its fermentative ability through a process of selection, using a single spore isolation procedure. Twenty single conidiospore isolations were made, using a modification of the Chambers (1922) technique with the micromanipulator modification of Wright and McCoy (1927). In isolating spores, the suspension of spores was allowed to incubate for about 3 hours, and the swollen but not yet germinated spores were picked for isolation; by this method a maximum percentage of germinated spores was obtained. The single-spore-derived cultures (from parent strain no. 62) were studied on the synthetic medium of Doelger and Prescott (1934), with the addition of 0.16 mg of iron and 0.003 mg of manganese per liter of medium.

The results in table 1 indicate that some stimulation, apparently due to the isolation procedure, was observed in almost all the cultures; no marked increase in fermentative ability was achieved and maintained in subsequent studies, however.

Unless otherwise specified, strain 72 of *Aspergillus niger* was employed in the subsequent studies on beet molasses media.

Development of a Beet Molasses Medium

Proximate chemical analyses of beet molasses revealed a comparatively high percentage of metals, particularly iron. Since the fundamental research of Currie (1917), it has been apparent that excess quantities of many metals may markedly inhibit the formation of citric acid by *A. niger* strains. Preliminary studies confirmed this fact: when untreated beet molasses was tested in the fermentation, a heavily sporulated mycelium mat was formed by the mold and relatively poor yields of citric acid were obtained. It was concluded, therefore, that the inhibiting factor in beet molasses might well be an excess quantity or an unfavorable balance of metallic ions, and the problem was approached from this point of view.

In the wine industry, potassium ferro- and ferricyanide frequently have been used to remove iron from the product (Creuss, 1934). Ferrocyanide was used for several years by Mr. William Eisenman of the Heyden Chemical Corporation for the processing of molasses in citric acid fermentations, and the authors wish to acknowledge their indebtedness to him for a statement of the procedure which he employed. A systematic study of various factors which might affect the treatment of molasses with ferro- and ferricyanide was undertaken.

Preliminary development of the medium was made using a single sample of beet molasses, no. 1. Optimum conditions for treatment of this molasses sample, which in many respects is similar to the procedure of Eisenman, were found to be as follows: To 340 g of beet molasses partially diluted were added 0.60 g of potassium ferrocyanide (in solution), and the whole was made to 1 liter with distilled water. Ten g of diatomaceous earth were added and mixed thoroughly. The medium thus prepared was allowed to stand overnight in a graduated cylinder or similar container at approximately 6 C. The medium was placed in the fermentation containers and autoclaved at 120 C for 15 minutes. The final medium thus prepared contained approximately 15 per cent sugar.

A total of four samples of Straighthouse beet molasses from different localities were treated with ferrocyanide. All of the samples gave satisfactory citric acid yields, although optimum conditions were established for only one sample, no. 1. The ferrocyanide level, pH, and sterilization conditions required for maximum yields with the different samples vary considerably. Thus, table 2 gives yields obtained with the four samples of molasses sterilized and unsterilized, in each case with the pH unadjusted and the ferrocyanide treatment optimum for the no. 1 molasses. Under these conditions, molasses no. 1 gave better results when sterilized, the other three apparently giving better results in an unsterile condition. No trouble was encountered from contamination of the unsterile media in the course of the fermentation, presumably because of the high concentration of acid formed. Further evidence of the necessity for determining the optimum treatment for a given lot of molasses in large-scale production became apparent in the case of molasses no. 2. The pH of this sample was 8.4, as compared to the nearly neutral reactions of the other samples. By adjusting the pH of this sample before ferrocyanide treatment to an optimum, pH 6.0, and sterilizing the medium, total acidity yields as citric were increased from 90.5 to 101.5 per cent, and citric acid yields, from 44.8 to 54.0 per cent.

TABLE 2

Effect of sterilization in the preparation of beet molasses media using different molasses samples

MOLASSES SAMPLE NO.	STERILIZATION	PERCENTAGE YIELDS, 10 DAYS			
		Total acidity as citric	Citric acid	Oxalic acid	Citric acid: total acidity*
1	+	102.1	58.8	39.1	60.0
	−	95.5	45.4	45.1	50.2
2	+	90.5	44.8	41.0	52.2
	−	69.5	47.8	19.6	70.9
3	+	37.5			
	−	86.6	53.6	29.6	64.4
4	+	30.7	18.5	11.0	62.7
	−	77.5	44.2	29.3	60.2

* Citric acid: total acidity $= \frac{\text{citric acid}}{\text{citric acid} + \text{oxalic acid}} \times 100.$

The relationship between total acidity yields and the amounts of potassium ferro- and ferricyanide added as precipitants to molasses no. 1 is illustrated in figure 1. An excess of the ferrocyanide apparently was somewhat less serious than an inadequate quantity, although the range of the optimum level was rather narrow. The optimum amount for 340 g of molasses sample no. 1 was about 0.60 g; the percentage of the citric acid yield as well as the total acidity yield was found to be maximum at this level. Although slightly higher yields were obtained with ferrocyanide, the ferricyanide treatment gave nearly equal yields and did not exhibit such a narrow optimum level.

Effect of pH. The optimum pH level for the ferrocyanide treatment was investigated in the next series of fermentations and was found to be approximately 7.0. This result is in marked contrast to the effect of pH on the production of citric acid in synthetic media; in this case, the yields were maximum at low pH

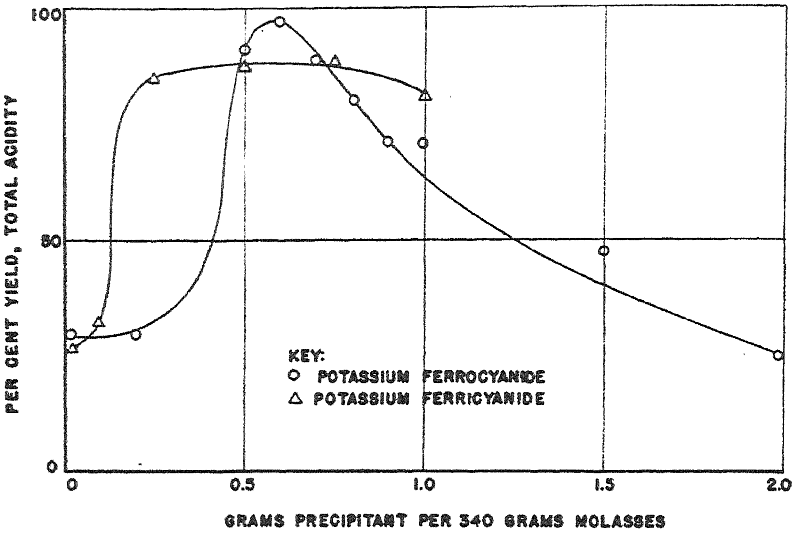

FIG. 1. EFFECT OF AMOUNTS OF POTASSIUM FERRO- AND FERRICYANIDE USED IN TREATMENT OF BEET MOLASSES

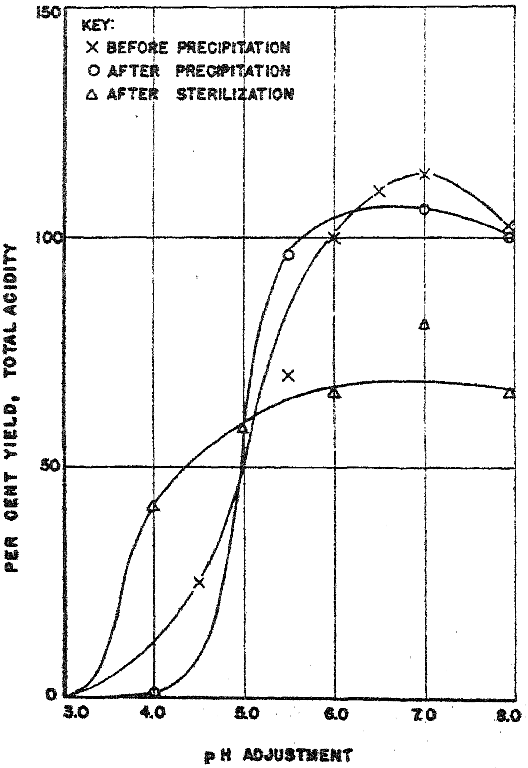

FIG. 2. EFFECT OF pH ADJUSTMENT IN FERROCYANIDE TREATMENT OF BEET MOLASSES

levels, often below 3.0. In the beet molasses media, yields of citric acid as well as total acidity were markedly better at the higher pH range; moreover, the mold spores failed even to germinate at a range below pH 4.0. Figure 2 shows the effect of varying the pH on total acidity yields with beet molasses no. 1. Variation in pH before ferrocyanide treatment of the medium, after treatment but before sterilization, and after both treatment and sterilization gave highest yields at a neutral reaction. The pH was routinely adjusted before ferrocyanide treatment and sterilization. In no case was pH adjustment made after the fermentation was started, nor was possible change in pH checked after the original adjustment. The germination of spores and mycelium formation were markedly inhibited at the lower pH values; there were no apparent differences in morphological characteristics of the mycelial mats between pH 5.0 and 8.0. Most of the other samples of beet molasses gave satisfactory results with the pH unadjusted throughout.

TABLE 3

Gross effect of different 30 C incubators and of different temperature levels within one incubator

INCUBATOR NO.	TEMPERATURE AVERAGE °C	PERCENTAGE LOSS IN VOLUME, 10 DAYS	PERCENTAGE YIELDS, 10 DAYS			
			Total acidity	Citric acid	Oxalic acid	Citric acid: total acidity
1	33	29	87.0	52.8	30.8	63.1
	30	22	85.4			
	28	19	83.0	47.0	31.8	59.6
	26	19	60.6			
	25	19	50.3			
2	30	44	79.0	39.7	35.2	53.1
3	30	54	73.8			
4	30	45	89.0	46.2	38.4	54.6
5	32	44	85.4	45.8	35.3	56.5
Room temp.	23	24	47.5			

Effect of temperature. Since various temperatures have been reported as optimum for the fermentation, it seemed desirable to check the influence of incubation temperature upon the fermentation of strain 72 on beet molasses medium. Composite results of the study are given in tables 3 and 4. A temperature of 30 C appeared to be optimal, and this temperature has been employed throughout the work. However, it was also indicated that marked differences in yields may occur using different incubators of apparently the same temperature, pointing to uncontrolled factors in incubation such as humidity, circulation, etc. An attempt to shorten the incubation period for the fermentation was made by using a preliminary incubation period of 4 days at a higher temperature followed by a secondary period at 30 C. Although spore germination and formation of the mycelium mat was accelerated by this method, no appreciable effects on yields were apparent (table 4).

Effect of inoculum. The influence of the size of spore inoculum upon the fermentation of strain 72 on beet molasses medium was checked. The results are given in table 5. A comparatively heavy seeding of spores was found to be necessary for satisfactory spore germination and mycelium formation. The heavier inocula served primarily to shorten the time of incubation necessary. The no. 2 suspension was employed as the standard inoculum for experimental work, with approximately 1 ml of the suspension per 50 cm^2 surface area of medium.

TABLE 4

Effect of preliminary incubation at a higher temperature with a secondary incubation at a lower temperature

PRELIMINARY INCUBATION, °C	SECONDARY INCUBATION, °C	PERCENTAGE LOSS IN VOLUME, 10 DAYS	PERCENTAGE YIELDS, TOTAL ACIDITY			PERCENTAGE YIELDS, 10 DAYS		
			8 days	10 days	12 days	Citric acid	Oxalic acid	Citric acid: total acidity
30	30	42	84.0	91.4	99.9	45.0	41.9	51.8
33	33	57	81.5	84.0	86.5	43.6	36.4	54.5
37	37	65	74.6	74.6	73.3	39.0	31.8	55.1
33	30	46	86.0	89.7	96.6	47.1	38.3	55.1
37	30	49	83.2	91.8	96.6	48.6	38.9	55.6

TABLE 5

Effect of amount of spore inoculum

SPORE SUSPENSION			PERCENTAGE YIELDS, TOTAL ACIDITY			
Dilution number	Density (2-log G)*	Number of spores per ml	6 days	8 days	10 days	12 days
1	4 × 1.824	108,800,000	69.2	80.6	84.9	84.2
2	2 × 1.824	54,400,000	56.2	68.8	73.1	88.6
3	1.824	27,200,000	46.4	60.2	77.1	71.2
4	0.930	13,600,000	43.3	62.2	68.4	76.3
5	0.542	6,800,000	44.8	64.9	57.0	62.8
6	0.274	3,400,000	31.8	56.2	58.6	69.5

* Evelyn photometer, 420 mμ filter.

Fermentation in Shallow Pans

Plant fermentation conditions were simulated by the employment of shallow aluminum pans (36 x 25.5 x 7.5 cm) containing 5 liters of medium to give a 5-cm depth. The composite results of a number of experiments conducted in these pans are given in table 6. In all cases the fermentation period was 10 days. The yields in general appeared to be fairly uniform. In one instance, unusually high yields of citric acid, as well as a high proportion of citric acid to total acidity, were obtained; however, these results were not consistent and pointed to uncontrolled factors. Yields of approximately 45 to 50 per cent citric acid were obtained consistently on a 5-cm depth of beet molasses medium with a moderately high concentration of sugar in the substrate. When high yields were obtained in pans with strain 72 on beet molasses medium, large amounts of a crystalline material were deposited. Chemical analysis indicated this material to be potassium tetraoxalate, $KH_3(C_2O_4)_2.2H_2O$. Adequate dilution and heating of the fermented liquor containing these crystals was necessary before sampling for analyses.

TABLE 6

Fermentation in shallow aluminum pans

EXP. NO.	PAN NO.	STRAIN NO.	PERCENTAGE SUGAR IN MEDIUM		PERCENTAGE LOSS IN VOLUME, 10 DAYS	PERCENTAGE YIELDS, 10 DAYS			
			Before fermentation	After fermentation		Total acidity	Citric acid	Oxalic acid	Citric acid: total acidity
1	1	62	15.0	4.8	24	40.8	30.6	9.3	76.8
	2	72	15.0	2.9	22	94.6	65.2	26.4	71.3
	3	72	15.0	2.3	32	103.1	72.0	27.9	72.0
2	1	72	14.8		50	91.6	45.7	41.2	52.6
	2	72	14.8		44	92.8	47.3	40.9	53.6
3	1	72	14.3	2.7	30	89.6	43.8	41.3	51.3
	2	72	14.3	2.1		93.9	46.8	42.5	52.4
4	1	72	15.0	1.5	43	94.2	45.6	43.3	51.3
	2	72	15.0	1.6	42	95.6	47.4	43.1	52.4
5	1	72	13.6	1.2	49	100.2	50.0	45.3	52.4
	2*	72	13.6	4.6		31.6	20.7	9.8	67.8
6	1	72	16.0		34	95.3	49.8	40.8	54.9

* Mycelium heavily sporulated.

ACKNOWLEDGMENT

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ADDENDUM

Since this manuscript was submitted, it has been brought to our attention that a similar process for the production of citric acid from beet molasses was in use in Germany during the war. A detailed account may be found in two reports by the British Intelligence Sub-Committee, 32 Bryanston Square, W.1, London: Chemische Fabrik Joh. A. Benckiser, G.M.B.H., Manufacture of calcium citrate, B.I.O.S. Final Report No. 489; and Production of citric acid at the factory of Joh. A. Benckiser, Ladenburg, near Heidelberg, B.I.O.S. Final Report No. 220.

SUMMARY

A study was made of beet molasses treated with potassium ferro- or ferricyanide for use as a substrate in mycological citric acid production. A selected strain of *Aspergillus niger* was used. Optimum conditions of precipitation, pH, incubation temperature, and inoculum were established for one beet molasses sample, although samples from several other sources also gave satisfactory results with this method. In shallow pans, simulating conditions in industry, yields of citric acid of approximately 50 per cent of the available sugar (calculated as sucrose) were attained.

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FACTORS AFFECTING THE VIABILITY OF SERRATIA MARCESCENS DURING DEHYDRATION AND STORAGE

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The preservation and storage of nonsporeforming bacteria have long been problems in the laboratory and in industry. Storage of liquid cultures for long periods of time has been found unsatisfactory with most species. Cultures dried under carefully controlled conditions have remained viable over long storage periods, but the percentage of surviving cells has usually been very low. This study was undertaken to determine conditions which would result in a high percentage of survival during dehydration and storage of an organism that is easily killed by drying.

A search of the literature revealed very few references to quantitative data on the survival of dried vegetative bacteria. The review by Rahn (1945) refers to some work in which quantitative data were obtained, but survivals were very low in most instances.

Rogers (1914) showed that powders of high viable cell count could be produced by spray-drying milk cultures of lactic streptococci or by drying such cultures from the frozen state under vacuum. On the assumption that the milk cultures contained one billion cells per ml, from a calculation of Rogers' results with *Streptococcus lactis*, giving consideration to the solids content of the skimmed milk, it would appear that he obtained survivals approaching 100 per cent in several samples of powder in which viable counts of over 10 billion per gram were observed. Rogers' experiments on the drying of cultures of *Lactobacillus bulgaricus* and the legume bacteria indicated that survivals of about 10 per cent were obtained with these nonsporeforming rods. Rogers also found that powders containing up to 1.39 per cent moisture stored satisfactorily at 17 C but not at 30 C. Higher survivals were observed in powders stored under vacuum than in those stored in air, oxygen, nitrogen, hydrogen, or carbon dioxide for a period of 4 months.

Differences in resistance to drying among various species of nonsporing bacteria were found by Stark and Herrington (1931). *Streptococcus lactis* and *Streptococcus paracitrovorous* were found to be more resistant than yeast and staphylococci, which were in turn more resistant than *Escherichia coli* and *Lactobacillus acidophilus*. Exposure of the dried cultures to oxygen resulted in a rapid loss of viability in all cases.

The work of Miller and Schad (1943) emphasizes the importance of protecting dried bacteria from sunlight. It was found that meningococci dried on various surfaces were rapidly killed by direct sunlight, and even diffuse sunlight passing first through window glass and then through pyrex glass caused a rapid loss of viability.

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A very important contribution was made by Stamp (unpublished data), who found that the percentage of survival of various delicate bacteria could be greatly increased by adding ascorbic acid to the cell suspensions just before they were dried. Small drops of suspensions prepared with nutrient gelatin and ascorbic acid were placed on paraffined filter paper and dried in the presence of P_2O_5 under vacuum. Not only were the initial survivals greatly increased, but the effect of ascorbic acid was even more striking after the samples had been stored for long periods of time. Stamp found that glutathione could not be substituted for ascorbic acid, and that plain gelatin would not replace nutrient gelatin. Maximum survival could not be obtained with less than 10 per cent gelatin. Much higher survivals resulted when suspensions were dried over P_2O_5 than when they were dried from the frozen state. Stamp's original work was done in England. In a few experiments performed in this laboratory, he was unable to obtain high survivals with *Serratia marcescens*, but the beneficial effect of ascorbic acid was clearly demonstrated.

MATERIALS AND METHODS

In the preliminary work, the drying method described by Stamp (unpublished) was used. Concentrated cell suspensions prepared by taking up the cells centrifuged from liquid culture in small volumes of diluent composed of 0.2 per cent Difco gelatin, 0.725 per cent $NaH_2PO_4 \cdot H_2O$, and 0.37 per cent Na_2HPO_4 at pH 6.5 were fortified with various materials. The final suspensions usually contained between 20 and 60 billion viable cells per ml. Using a calibrated capillary dropping pipette controlled by means of a small rubber bulb, droplets of uniform size were placed on paraffined filter paper. These were dried in the presence of P_2O_5 in evacuated desiccators placed in a cold room at 4 C. After 24 hours, the vacuum was released, and the dry material in the form of disks was stored at room temperature and atmospheric pressure in the presence of P_2O_5. Quantitative survivals were determined by comparing the number of viable cells per drop of suspension with the number of viable cells per dry disk. Ten drops or disks were used in making each count. The dilutions were prepared in a gelatin-phosphate diluent of the composition given above, and standard plating techniques were used in determining counts. The agar medium was composed of 1.0 per cent Difco tryptone, 0.5 per cent Difco yeast extract, 0.5 per cent glucose, and 1.5 per cent agar. The plates were incubated at 34 C for 18 to 24 hours.

The second method studied was that of freeze-drying, or lyophilization as it will be referred to in this paper. The apparatus employed in this phase of the work was of conventional design, consisting of a condensing chamber cooled with dry ice and alcohol. Two manifolds, bearing ten outlets each, were connected to the condenser. Short pieces of rubber pressure tubing were used to connect the manifold outlets and the necks of the ampules containing the material to be lyophilized.

Fortified cell suspensions containing between 100 and 150 billion cells per ml were carefully measured into glass ampules. In most instances 1-ml samples were placed in 10-ml ampules. When larger samples of powder were required for moisture determinations, larger ampules containing greater volumes of suspension were used. The suspension was shell-frozen by inserting the neck of the ampule in the chuck of an electric stirring motor and spinning it at high speed while the ampule was immersed in a dry ice, alcohol bath at −50 to −60 C. When the suspension was completely frozen, the ampule was immersed in an alcohol bath and attached to the lyophilizer. The temperature of the baths was held below the freezing point of the suspension during the drying period by means of dry ice. A high vacuum was maintained by means of a Cenco hypervac 20 vacuum pump connected to the condensing chamber by means of rubber pressure tubing. When all traces of ice had disappeared from the samples, the cold baths were removed and the vacuum was maintained for an hour with the ampules at room temperature. The ampules were sealed with a hand torch. In most instances, they were sealed under a vacuum of 2 to 5 microns Hg, but in a few cases they were allowed to fill with nitrogen before they were sealed. All samples were stored at room temperature in a dark place. The percentage of survivals was determined by comparing the count on the dry material in a given ampule with the count on an equivalent volume of the original cell suspension. Replicate ampules were prepared with each cell suspension tested in order that survivals could be determined at intervals during the storage period. Preliminary trials showed that good agreement could be expected among replicates.

Serratia marcescens was selected as the test organism because it is nonpathogenic, easily cultured, and yet very sensitive to dehydration. The culture employed was maintained on agar slopes of the composition given for the plating agar. It was transferred regularly and incubated at room temperature. The typical red pigment was produced under these conditions.

EXPERIMENTAL

The effect of nutrition on the resistance of the cells to drying was not studied in detail. A liquid medium composed of 1.0 per cent Difco tryptone, 0.5 per cent Difco yeast extract, 0.1 per cent glucose, 0.2 per cent Na_2HPO_4, and 0.1 per cent NaH_2PO_4 at pH 6.8 to 7.0 was found to be satisfactory. Aeration proved to be a very important factor in the production of resistant cells in this medium. When aerated by agitating vigorously on a shaking machine, liquid cultures of this organism yielded cells which were far more resistant to drying than cells produced without aeration. It was also found that cultures aerated at 30 to 34 C for a period of 18 to 24 hours were more resistant than cultures incubated for shorter or longer periods. Thus all cell suspensions used in this work were prepared by centrifuging the cells from 18- to 24-hour cultures aerated by shaking 1-liter volumes of the medium in 6-liter Erlenmeyer flasks.

With the drying method described by Stamp and *l*-ascorbic acid obtained from Merck and Company, a number of preliminary trials were made in which it was found that 0.5 per cent ascorbic acid was the optimum level. It was also found that when the fortified cell suspensions were adjusted to final pH values between 6 and 7 with N NaOH prior to drying, maximum survivals resulted.

Stamp's inability to demonstrate survivals of *S. marcescens* comparable to those which he had observed in England was believed to be due to differences in the nutrient gelatin used. To determine whether the difference was due to the gelatin, numerous trials were made to compare a number of gelatin samples. In concentrations varying from 1 to 10 per cent, no differences could be detected among the samples. Furthermore, it was found that gelatin could be replaced with good grades of white dextrin or of pectin without affecting the survival rate.

Attention was next directed to the effect of various nutrient materials on the survival rate. These materials were added to cell suspensions containing ascorbic acid and gelatin or dextrin. Of the various products tested, marmite (a yeast product manufactured in Great Britain) gave the greatest protection to the cells during and following the drying process. It was found that with this method of drying, the use of marmite made it possible to eliminate gelatin or dextrin because marmite contained colloidal materials in addition to other factors necessary for maximum survival. The results presented in table 1 show the individual and combined effects of ascorbic acid and marmite on survival.

TABLE 1

Effect of ascorbic acid and marmite on the survival of dry Serratia marcescens

FINAL CONC. OF ADDED MATERIAL	SURVIVAL (%) OF DRIED S. MARCESCENS AFTER		
	10 days	1 mo.	5 mo.
Control	<0.1	—	—
1.0% Marmite	0.3	—	—
0.5% Ascorbic acid	36.2	19.9	19.8
0.5% Ascorbic acid + 1.0% marmite	46.0	55.4	46.7

Throughout this work, fortifying materials were added as concentrated solutions calculated to give the desired final percentage of concentrations on a weight per unit volume basis. The volume of concentrated cell suspension used in preparing the samples was constant. When necessary, distilled water was added to adjust the final volume of the samples.

In table 2, the effect of increasing marmite concentration in the presence of 0.5 per cent ascorbic acid is shown. It should be re-emphasized that all disk samples were stored in air over P_2O_5 at room temperature.

Attention was next given to drying cell suspensions from the frozen state. Preliminary trials showed that, as with the disk method, very poor survival resulted when unfortified suspensions were dried. Counts made immediately after drying indicated that survivals of more than 5 per cent were never obtained, and in most instances less than 1 per cent of the cells survived. The protective effect of ascorbic acid and marmite was marked, but it was found that 0.5 per cent ascorbic acid was not optimum for maximum survival. This was probably due to the fact that more concentrated cell suspensions were employed than with the disk procedure. The effect of increasing ascorbic acid concentration in samples containing 2 per cent dextrin and 2 per cent marmite before drying is shown in table 3. The lyophilized samples were sealed under vacuum and stored at room temperature.

The excessive amount of ascorbic acid required to obtain high survivals, together with the marmite (a very hygroscopic material when dried), made lyophilization difficult. Two steps were taken which helped overcome this difficulty. Ascorbic acid was partially replaced with thiourea, and marmite was entirely replaced with inorganic ammonium salts.

In attempting to learn how ascorbic acid functions in protecting the cells when dehydrated, a number of reducing compounds and antioxidants were tried as substitutes for the vitamin. Of these, only two offered any degree of protection.

TABLE 2

Effect of marmite concentration on the survival of dry Serratia marcescens

MARMITE CONC.	SURVIVAL (%) OF DRIED S. MARCESCENS AFTER		
	15 days	1 mo.	3 mo.
%			
0	61	36	46
1	62	67	61
2	77	70	81
4	87	86	78
10	82	80	82

TABLE 3

Effect of ascorbic acid concentration on the survival of lyophilized Serratia marcescens

ASCORBIC ACID CONC.	SURVIVAL (%) OF LYOPHILIZED S. MARCESCENS AFTER			
	1 day	1 mo.	2 mo.	3 mo.
%				
0.00	12	3	—	—
0.25	21	—	20	18
0.50	39	28	32	22
1.00	61	53	49	—
2.00	74	81	63	68

Cysteine protected the cells as well as ascorbic acid, and thiourea, a cheap compound, offered considerable protection during the drying process but failed to maintain a high level of viability during storage. The latter compound has been shown by Kawereau and Fearon (1944) to be very effective in protecting vitamin C against oxidation by cupric ions. This suggested the possibility of increasing the effectiveness of ascorbic acid by adding thiourea to the cell suspensions. The effect of thiourea on the ascorbic acid level required to protect the lyophilized cells can be seen by comparing tables 3 and 4. The data shown in table 4 were obtained by lyophilizing suspensions containing 0.5 per cent thiourea, 2.0 per cent dextrin, and inorganic ammonium salts in addition to the indicated ascorbic acid levels. The substitution of ammonium salts for marmite will be discussed subsequently. These samples were easily lyophilized to yield a porous dry material.

The substitution of ammonium salts for marmite was the result of a study of the protective properties of the yeast product. A comparison of marmite and Difco yeast extract showed that the extract was completely lacking in the protective factor. It was then decided to attempt a fractionation of the marmite. With a continuous dialysis system similar to that described by Hanke and Koessler (1926), it was possible to remove all of the protective fraction by continuously dialyzing a 20 per cent marmite solution at pH 5.6 against distilled water. It was found that the protective fraction was present in the dialyzate. Continuous extraction of the dialyzate at pH 4.5 with ethyl ether for a period of 24 hours failed to remove the protective factor. Powdered marmite extracted with ethyl alcohol and *n*-butyl alcohol still retained most of its protective action. The evidence, although not complete, suggested that inorganic salts were responsible for the observed protection. Since it had been noticed that considerable ammonia was evolved from the dialyzate when it was adjusted to pH 9.0 with NaOH, the presence of inorganic ammonium salts was suspected. Cell suspensions were then prepared with 0.5 per cent NH_4Cl, 0.5 per cent $(NH_4)_2SO_4$, ascorbic acid, and dextrin. When lyophilized, these samples showed much higher survivals than the controls which contained marmite. It was then found that NH_4Cl alone

TABLE 4

Effect of thiourea on concentration of ascorbic acid required to protect lyophilized Serratia marcescens

ASCORBIC ACID CONC.	VIABILITY OF LYOPHILIZED S. MARCESCENS AFTER					
	1 day		15 days		2 mo.	
	Count ($\times 10^{12}$/g)	Survival (%)	Count ($\times 10^{12}$/g)	Survival (%)	Count ($\times 10^{12}$/g)	Survival (%)
%						
0.000	1.6	76	0.8	38	—	<0.1
0.125	1.3	75	1.0	60	1.2	70
0.250	1.3	82	1.0	65	1.1	67
0.500	1.9	100	1.2	72	1.5	89
1.000	1.6	93	1.4	79	1.3	72

TABLE 5

Effect of ammonium chloride concentration on the survival of lyophilized Serratia marcescens

NH_4Cl CONC.	SURVIVAL (%) OF LYOPHILIZED S. MARCESCENS AFTER	
	1 mo.	2 mo.
%		
0.00	17	15
0.10	64	47
0.25	86	83
0.50	95	86
0.75	100	86
1.00	93	89
2.00	62	63

was more satisfactory than its mixture with the sulfate. To determine the optimum concentration of NH_4Cl, increasing amounts of the salt were added to cell suspensions fortified with 0.5 per cent ascorbic acid, 0.5 per cent thiourea, and 1.0 per cent dextrin. Survival results on the lyophilized samples stored under vacuum at room temperature are presented in table 5.

Although no extensive study of the effect of other inorganic salts was made, a protective action was demonstrated with NaCl. However, the degree of protection found with the optimum concentration of NaCl was not so great as that with NH_4Cl.

At this point, it was known that high survivals and satisfactory storage could be attained by drying *S. marcescens* suspensions containing dextrin, ascorbic acid, thiourea, and NH_4Cl. The approximate optimum concentration of NH_4Cl had been determined, but the optimum thiourea-ascorbic-acid balance was not known. To determine this, a series of suspensions was prepared in which four levels of thiourea were tested in combinations with five different concentrations of ascorbic acid. Zero levels were omitted in these experiments since the results at these levels had been obtained in previous tests. Each suspension contained 2.0 per cent dextrin and 0.5 per cent NH_4Cl. The samples were lyophilized, sealed under vacuum, and stored at room temperature. After a period of 2 months, the survivals were as indicated in table 6.

TABLE 6

Effect of thiourea-ascorbic-acid balance on the percentage of survival of lyophilized Serratia marcescens stored 2 months*

ASCORBIC ACID CONC.	THIOUREA CONC. (%)			
	0.10	0.25	0.50	1.00
	Survival (%)			
%				
0.10	54	77	65	38
0.25	72	95	84	68
0.50	82	87	100	84
0.75	100	100	100	80
1.00	97	100	100	77

* In a few instances the survivals were slightly in excess of 100 per cent because of the inherent plating error. These figures have been rounded off at 100 per cent.

Colloidal materials alone had little beneficial effect in the drying of *S. marcescens*, yet they were essential for maximum survival with ascorbic acid, thiourea, and NH_4Cl. Dextrin was preferred to gelatin because of its lower viscosity and easy reconstitution after drying.

In the preparation of stable, dried bacteria, three general considerations are involved. First, it is essential that the cells be produced under conditions that give maximum resistance to dehydration. Second, a suspending medium must be developed which will protect the organisms during and following the dehydration process. Although this phase of the problem was the most critical, the third consideration, storage conditions, was found to be important.

Since it is known that bacteria are killed by certain radiations, samples were stored in the dark. The effect of storage temperature was not studied. All samples were stored at room temperature, and satisfactory survivals have been observed for the reported storage periods. Lower storage temperatures would undoubtedly result in higher survivals after long periods of time. The effect of the temperature to which the lyophilized samples were exposed after all ice crystals had disappeared and before the ampules were sealed was determined. In this experiment, the samples were lyophilized until all visible ice crystals had disappeared. Then while the ampules were still under a high vacuum, a set of five was heated in water at 40 C for 2 hours, a second set was heated at 50 C for 2 hours, and a third set at 70 C for 2 hours. The controls were under vacuum at room temperature for 6 hours. Each of the other three sets of samples was at room temperature for 4 hours in addition to the 2-hour heating period. The moisture content was then determined on the basis of the weight lost by the samples when heated to 80 C for 6 hours in a vacuum of 29 inches of Hg. Survivals of the samples sealed and stored under an atmosphere of nitrogen are shown in table 7.

TABLE 7

Effect of finishing temperature and moisture content on the survival of lyophilized Serratia marcescens held under an atmosphere of nitrogen

FINISHING TEMP.	MOISTURE	SURVIVAL (%) OF LYOPHILIZED S. MARCESCENS AFTER		
		1 day	14 days	42 days
	%			
25 C	2.10	91	82	57
40 C	1.52	83	71	43
50 C	1.09	74	75	42
70 C	0.42	67	65	31

TABLE 8

Viability of lyophilized Serratia marcescens stored in air, nitrogen, and vacuum

STORED IN	SURVIVAL (%) OF LYOPHILIZED S. MARCESCENS AFTER 49 DAYS
Air	9
Vacuum	99
Untreated nitrogen	28
Treated nitrogen	26

The deleterious effect of oxygen on dried bacteria has been observed by several workers. It was also observed in the present study. Although material dried in the form of disks stored very well in air, lyophilized samples showed a rapid loss of viability. This difference is believed to be due to the relatively small surface area exposed in the case of the disks as compared to the exposed surface in the porous lyophilized material. In most cases the lyophilized samples were sealed under vacuum. In a few instances, samples were sealed under an atmosphere of ordinary tank nitrogen. Storage results on those samples were always poor. It was then recalled that Rogers (1914) had found that dried lactic streptococci stored much better under vacuum than under nitrogen. It was difficult to understand why this should be true unless the nitrogen was contaminated with other gases. Consequently, an experiment was performed in which replicate samples of a lyophilized *S. marcescens* suspension were stored under different conditions. One lot of these samples was sealed under a high vacuum, the second lot was sealed under an atmosphere of air, the third under an atmosphere of tank nitrogen, and the fourth under an atmosphere of "purified" nitrogen. The "purification" was carried out by passing the nitrogen through pyrogallol, saturated $Ba(OH)_2$ solution, and anhydrous $CaSO_4$ (dririte) to remove oxygen, carbon dioxide, and moisture before the nitrogen entered the ampules. The storage data are presented in table 8.

These results show the deleterious effect of nitrogen on the dried cells. Time did not permit further investigation of this problem.

SUMMARY

To obtain maximum survival of *Serratia marcescens* during dehydration and storage, the following conditions proved most satisfactory:

The cells were harvested at the end of the logarithmic growth phase from an aerated culture (18 to 24 hours at 30 to 34 C). The cell concentrate was then mixed with a solution containing ascorbic acid, thiourea, NH_4Cl, and dextrin at pH 6 to 7. The optimum concentrations of these constituents were approximately 0.5, 0.5, 0.5, and 2.0 per cent, respectively, when dehydration was accomplished by lyophilization.

The viability of lyophilized material was lost rapidly when the material was stored in air or nitrogen. A high percentage of survival was maintained, however, when samples were stored in a high vacuum.

Survivals of 100 per cent and counts ranging up to 5×10^{12} per gram of dried material were obtained under optimum conditions of drying and storage.

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THE INHIBITION OF BACTERIA BY THIOPYRIMIDINES

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We have reported (Strandskov and Wyss, 1945) that thiouracil and thiothymine inhibit bacterial growth and metabolism and that the inhibition is reversed by the corresponding metabolite analogue. This effect of thiouracil on *Escherichia coli* has also been observed by Roepke and Jones (cited by Roblin, 1946). Hitchens, Falco, and Sherwood (1945) failed to observe inhibition of *Lactobacillus casei* by 10 mg per cent thiothymine when tested in the presence of 0.1 mg per cent thymine. The experiments reported here show the quantitative aspects of the inhibitions and indicate their usefulness as methods of assay for small quantities of pyrimidines.

METHODS

The casein hydrolyzate medium of Landy and Dickens (1942) was used for growing *L. casei*. *E. coli* was grown on a simple mineral salts asparagine glucose medium. The *L. casei* inoculum was grown in yeast extract broth, separated by centrifuging, washed twice with saline, and inoculated into the test medium so that the initial count was 100,000 cells per ml. Acid production was measured by titration after 72 hours' incubation at 37 C. The *E. coli* inoculum was grown in the simple medium, washed twice, and inoculated at a level of 1,000 organisms per ml. The extent of growth was measured by 24-hour turbidity readings.

Uracil and yeast nucleic acid were obtained from Eastman. Thiouracil (2-thio 6-oxy pyrimidine), thiothymine (2-thio 5-methyl 6-oxy pyrimidine), and the 4-methyl thiouracil (2-thio 4-methyl 6-oxy pyrimidine) were prepared by R. R. Joiner of the Chemical Research Division of Wallace and Tiernan Products. These compounds were dissolved in sterile water and sterilized by minimal heating immediately before use. The folic acid was supplied by a liver concentrate of vitamin Bc obtained from Parke, Davis and Co.

RESULTS

Under the conditions stated *E. coli* made no measurable turbidity in 24 hours in the presence of 25 mg per cent thiouracil. The addition of uracil at a metabolite to inhibitor ratio of 1:100 permitted growth equal to the control, but no growth occurred in 24 hours at a ratio of 1:500 (table 1). After 48 hours' incubation, however, growth was observed even in the tube containing 50 mg per cent thiouracil. Filtrates from such cultures were found to retain their initial inhibitory activity, indicating that the thiouracil was not converted to an inactive form. Nor were the bacteria isolated from the old cultures significantly more resistant to thiouracil than the original culture. Continued transfer on the thiouracil medium did result in resistant cultures which retained their resistance even after several transfers in a medium devoid of the inhibitor.

TABLE 1

The effect of uracil on the bacteriostatic action of thiouracil on Escherichia coli

THIOURACIL	URACIL	TURBIDITY, 24 HR
mg %	*mg %*	
0	0	0.2480
10	0	0.2291
25	0	0
50	0	0
50	0.1	0
50	0.2	0.0555
50	0.5	0.2174
50	1.0	0.2291

TABLE 2

The effect of uracil on the bacteriostatic action of thiouracil on Lactobacillus casei

THIOURACIL	URACIL	TITRATION, 72 HR, ML N/10 NaOH
mg %	*mg %*	
0	0	10.2
0.01	0	10.2
0.1	0	1.3
0.1	0.001	1.2
0.1	0.01	2.0
0.1	0.1	10.4
1.0	0.01	1.3
1.0	0.1	4.6
1.0	1.0	8.9

TABLE 3

Assay for uracil

MATERIAL	URACIL ADDED	URACIL FOUND
	per cent	*per cent*
Yeast nucleic acid	0	6.8
Yeast nucleic acid	1.0	7.8
Yeast nucleic acid	2.5	9.2
E. coli cells (dry)	0	1.5
E. coli cells (dry) thiouracil-resistant	0	1.45

L. casei was much more sensitive to thiouracil; even at low concentrations the inhibition was manifest after the 3-day incubation period. Table 2 shows that the acid production was completely inhibited at a metabolite to inhibitor ratio of 1:100, partially suppressed at a ratio of 1:10, and little affected at a ratio of 1:1.

By determination of the metabolite to inhibitor ratio it was possible to assay for uracil at concentration levels below 1 μg per ml. The material to be assayed was autoclaved for 3 hours with 30 per cent sulfuric acid, and most of the excess sulfate was removed with barium. The samples were neutralized with NaOH and filtered, and suitable aliquots were added to the medium containing thiouracil. The results obtained from comparison with the uracil controls are reported in table 3. Good recovery of uracil added before hydrolysis to yeast nucleic acid was obtained. The thiouracil-resistant *E. coli* cells contained no more uracil than did the parent culture. One liter of Seitz filtrate from each of these cul-

TABLE 4

The bacteriostatic action of thiothymine and 4-methylthiouracil on Lactobacillus casei

THIOTHYMINE	72-HR TITRATION N/10 NaOH
mg %	*ml*
0 (Uninoculated)	2.4
0	7.2
1	6.9
2	6.1
5	4.5
10	3.1
25	2.4
4-METHYL THIOURACIL	
25	7.7
50	7.4

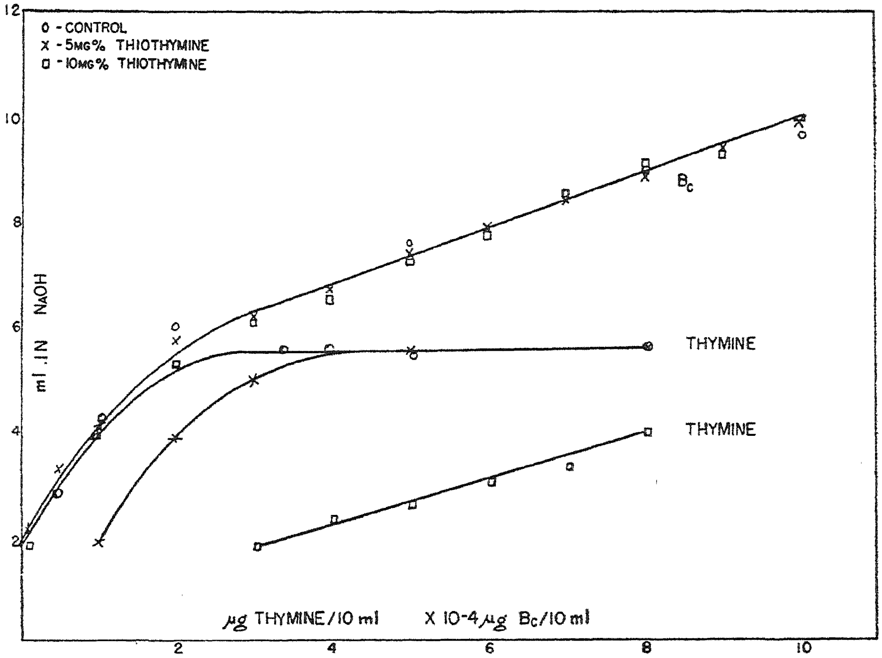

FIG. 1. RELATIONSHIP of THYMINE and VITAMIN Bc AS INDICATED BY THIOTHYMINE INHIBITION

tures was evaporated to dryness, hydrolyzed, and tested for uracil activity, but the amounts present in both cases were below the sensitivity of the method.

Thiothymine showed little or no bacteriostatic activity for *E. coli*, but inhibited growth and acid production of *L. casei* when thymine was supplied as a substitute for folic acid. Table 4 shows the inhibitory effect in the presence of 0.02 mg per cent of thymine. The specificity of the thymine compound is emphasized by the complete lack of inhibition of 4-methyl thiouracil, a compound which differs from thiothymine only in that it is the 4-methyl rather than the 5-methyl member of the series. The quantitative aspects of the relationships are shown in figure 1. In the presence of vitamin Bc thiothymine has no effect upon acid production. The standard curve for thymine, in agreement with the results of other workers, gives a maximum acid production considerably below that given by vitamin Bc. Inhibition by 5 mg per cent thiothymine was completely reversed by 0.05 mg per cent thymine. From the curve it is evident that the action of 10 mg per cent thiothymine will be almost completely reversed by 0.1 mg per cent thymine. In both cases the metabolite to inhibitor ratio is 1:100.

DISCUSSION

These additions to the already imposing list of inhibitory analogues of metabolites have, in common with most of the others reported, little chemotherapeutic significance. The marked physiological action of thiouracil and the low activity of thiothymine bars them from consideration as useful antibacterial agents. Such studies do add new tools for the study of biological processes. The failure of thiothymine to inhibit *L. casei* in the presence of vitamin Bc is strong evidence that thymine precedes Bc in some synthetic process. The failure of thiouracil-resistant organisms to synthesize uracil or to destroy thiouracil suggests an alternative mechanism for resistance. Such an explanation is necessary for certain sulfonamide-resistant strains which fail to synthesize increased amounts of *p*-aminobenzoate. If one accepts the Michaelis-Menton equations for competitive inhibition

$$\mathrm{E} + \mathrm{S} \leftrightarrow \mathrm{ES} \leftrightarrow \mathrm{E} + \mathrm{P}$$
$$\mathrm{E} + \mathrm{I} \rightleftharpoons \mathrm{EI}\ \text{(inactive)}$$

as the explanation for this effect, it is evident that, when the destruction of inhibitor and the increase in substrate concentration are ruled out, there remains only an increase in enzyme concentration which would account for the resistance phenomenon. Such an explanation was suggested by Yudkin (1938), whose theory on adaptive enzyme formation predicts the production of enzyme by substances which combine with it but are not broken down, i.e., by reversible inhibitors.

SUMMARY

The inhibition of bacterial growth and metabolism by thiouracil is reversed by uracil. The competitive ratios are such that under certain conditions a satisfactory uracil assay can be accomplished. Organisms rendered resistant to thioura-

cil action contain no more uracil than the parent strain from which they were derived. Thiothymine functions as a competitor for thymine but is completely inactive in a medium containing folic acid.

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THE OXIDATION OF PENTOSES BY PSEUDOMONAS

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The production of acid when pentoses are supplied as carbon sources for members of the genus *Pseudomonas* has been reported by several investigators (Chernomordik, 1939; Sears and Gourley, 1928), but apparently no attempt has been made to identify the products. Foster (1944), studying manometrically the oxidation of *d*-ribose and *d*-arabinose by *P. riboflavina*, found that only about 40 per cent of the oxygen theoretically required for the complete oxidation to carbon dioxide was consumed. He concluded that the remainder of the sugars was assimilated, there being no indication of acid accumulation.

In almost all papers dealing with the fermentation of pentoses by bacteria, the configurations of the sugars studied are not indicated. It is generally assumed that the sugars studied were the common, naturally occurring enantiomorphs. Thus, except for arabinose, which most commonly occurs in the *l*-form, the pentoses studied were probably of the *d*-series.

The present investigations were made to determine whether there were differences in the ability of various species of *Pseudomonas* to utilize *d*- and *l*-arabinose, to identify the acids produced when various pentoses are oxidized by *Pseudomonas*, and to obtain yield data.

MATERIALS AND METHODS

In order to obtain data on the nature of the products of the metabolism of pentoses by *Pseudomonas* and to get yield data, 100-ml cultures were aerated with 100 ml of filter-sterilized air per minute in 250-ml test tubes equipped with aerator stones which dispersed the air in fine bubbles. All cultures were incubated at 30 C. The corn steep liquor, which was the commercial grade product widely used in industrial fermentation processes, contained about 50 per cent solids, and the mineral nutrients supplied were of cp quality. The basal nutrient solution used in the 100-ml cultures contained 2 g urea, 0.6 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, and 5 ml corn steep liquor per liter. Three drops of soybean oil were added to each culture to prevent excessive frothing. At the time of inoculation, sufficient sterile $CaCO_3$ was added to neutralize the pentonic acid which might be formed if quantitative conversion of the sugar to a pentonic acid took place. The $CaCO_3$ was sterilized dry. Since preliminary experiments with *d*-xylose had shown the formation of furfural in toxic concentration in culture media sterilized 20 minutes at 15 pounds' steam pressure, all culture media used in these experiments were sterilized by filtration through Seitz sterilizing pads.

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All cultures were inoculated with 6 ml of 24-hour broth cultures which contained 0.2 per cent pentose, 0.3 per cent tryptone, and 0.3 per cent yeast extract.

The *d*-arabinose used in these experiments, supplied by Dr. Ray Hann, National Institute of Health, Bethesda, Maryland, was prepared by degradation of gluconic acid. The *l*-arabinose and *d*-xylose were of a pure reagent grade. The *d*-ribose, for which polarimetric examination indicated a purity of 99.7 per cent, was obtained through the courtesy of Dr. J. A. Aeschlimann.

All cultures used were from the culture collection of the Fermentation Division, Northern Regional Research Laboratory, and the identifying numbers used here are those of this collection.

TABLE 1

The oxidation of d-arabinose by Pseudomonas

CULTURE	*d*-ARABINOSE* CONSUMED	*d*-ARABONIC ACID		
		Produced†	Yield based on *d*-arabinose	
			Consumed (theoret.)‡	Supplied (weight)§
	g/culture	*g/culture*	*per cent*	*per cent*
P. fragi 73	4.8	1.20	22.6	25.0
P. graveolens 14	2.7	0.73	24.6	15.2
P. synxantha 79	2.5	1.20	22.6	25.0
P. vendrelli 23	1.4	0.73	52.0	15.2

* 4.8 g *d*-arabinose supplied per culture, duration 7 days.

† Calculated from data on calcium in solution.

‡ Theoretical yield: 1.107 g *d*-arabonic acid per g *d*-arabinose.

§ $\frac{\text{Grams } d\text{-arabonic acid produced}}{\text{Grams } d\text{-arabinose supplied}}$.

EXPERIMENTAL

The oxidation of *d*-arabinose was first studied. In table 1 are data for *P. fragi* 73, *P. graveolens* 14, *P. synxantha* 79, and *P. vendrelli* 23, which utilized a substantial proportion of the 4.8 grams *d*-arabinose supplied per 100 ml and produced considerable quantities of a soluble calcium salt. This was identified as calcium *d*-arabonate by preparation of the phenyl-hydrazide of the free acid (mp 213) (Glattfeld, 1913). A sample, mixed with synthetic *d*-arabonic phenylhydrazide melted at the same temperature. Other cultures which, while making considerable growth, either failed to oxidize the *d*-arabinose supplied in this experiment or failed to produce appreciable acid were *P. ovalis* 8, *P. schuylkilliensis* 9, *P. mildenbergii* 21, *P. fluorescens* 6, *P. putida* 13, *P. pavonacea* 24, and *P. mephitica* 75. The last four probably used the protein constituents of the media for growth. It is of interest that *P. ovalis* 8 oxidizes glucose to gluconic acid, and *P. schuylkilliensis* 9, *P. putida* 13, and *P. mildenbergii* 21 produce 2-oxogluconic acid from glucose in good yields (Lockwood, Tabenkin, and Ward, 1941).

Cultures of *P. fluorescens* 6, *P. fragi* 25, *P. mildenbergii* 21, *P. putida* 13, *P.*

synxantha 79, and *P. vendrelli* 23 were grown on 4.9 per cent *l*-arabinose nutrient solution. The first four species were harvested after 2 days' aeration, but the last two species oxidized the *l*-arabinose much less rapidly and were not harvested until the sixth day. At harvest, all cultures contained substantial amounts of a soluble calcium salt, which was in each case identified as calcium *l*-arabonate by preparation of the brucine salt (mp 152 C); when this was mixed with synthetic brucine *l*-arabonate, the melting point showed no depression. Nef (1907) reported the melting point of the brucine *l*-arabonate to be 155 C. As a further check on the identity of the material, the *l*-arabobenzimidazole derivative was prepared. This melted at 235 to 236 C, and when it was mixed with purely synthetic material, the resulting mixture melted at the same point (Moore and Link, 1940). Examination of X-ray diffraction patterns confirmed the identity of the benzimidazole derivatives of *l*-arabonic acids of synthetic and bacterial origin. Yields of *l*-arabonic acid are given in table 2.

TABLE 2

The oxidation of l-arabinose by Pseudomonas

CULTURE	AGE AT HARVEST	*l*-ARABINOSE CONSUMED*	*l*-ARABONIC ACID		
			Produced†	Yield based on *l*-arabinose	
				Consumed‡ (theoret.)	Supplied§ (weight)
	days	*g/culture*	*g/culture*	*per cent*	*per cent*
P. fluorescens 6	2	4.9	1.38	25.4	28.2
P. fragi 25	2	4.9	1.75	32.2	35.7
P. mildenbergii 21	2	4.9	1.41	26.0	28.8
P. putida 13	2	4.9	2.34	43.1	47.8
P. synxantha 79	6	4.3	0.86	18.0	17.5
P. vendrelli 23	6	4.9	2.22	40.9	45.4

* Four and nine-tenths g *l*-arabinose supplied per culture.

† Calculated from data on calcium in solution.

‡ Theoretical yield: 1.107 g *l*-arabonic acid per g *l*-arabinose.

§ $\frac{\text{Grams } l\text{-arabonic acid produced}}{\text{Grams } l\text{-arabinose supplied}}$.

Cultures of *P. fluorescens* 6, *P. ovalis* 8, *P. putida* 13, *P. graveolens* 14, *P. mildenbergii* 21, and *P. fragi* 25 grown on 6.7 per cent *d*-xylose nutrient solution for 6 days contained much soluble calcium salt, which was found to be calcium *d*-xylonate. The identity of the compound was established by preparation of the brucine salt (mp 176 C) (Nef, 1914). Yield data are presented in table 3.

In table 4 are presented the data for experiments in which fermentations were conducted on 5.9 per cent *d*-ribose. Cultures of *P. fluorescens* 6, *P. fragi* 25, *P. graveolens* 14, *P. mephitica* 75, *P. mildenbergii* 21, *P. ovalis* 8, *P. pavonacea* 24, *P. putrifaciens* 76, *P. synxantha* 79, and *P. vendrelli* 23 were employed. When the reducing action toward Shaffer-Hartmann copper reagent (Shaffer and Hartmann, 1921) was nil, or after 9 days, cultures were harvested. *P. pavonacea* 24 consumed little *d*-ribose, and little $CaCO_3$ was dissolved in either this culture or in the culture of *P. mephitica* 75. *d*-Ribonic acid was identified as the product of the oxidation of *d*-ribose by preparation of the benzimidazole derivative which melted at 191 C (Dimler and Link, 1943); when this was mixed with a sample of synthetic material, there was no depression of the melting point. The identity

TABLE 3

The oxidation of d-xylose by Pseudomonas

CULTURE	*d*-XYLOSE* CONSUMED	*d*-XYLONIC ACID		
		Produced†	Yield based on *d*-xylose	
			Consumed‡ (theoret.)	Supplied§ (weight)
	g/culture	*g/culgure*	*per cent*	*per cent*
P. fluorescens 6	5.9	4.59	70.4	68.0
P. ovalis 8	5.9	2.66	41.2	39.5
P. putida 13	5.4	4.40	73.4	65.3
P. graveolens 14	6.2	3.78	55.5	56.2
P. mildenbergii 21	6.3	4.87	70.1	72.4
P. fragi 25	5.5	1.89	31.0	28.1

* Six and seven-tenths g *d*-xylose supplied per culture, duration 6 days.

† Calculated from data on calcium in solution.

‡ Theoretical yield: 1.107 g *d*-xylonic acid per g *d*-xylose.

§ $\frac{\text{Grams } d\text{-xylonic acid produced}}{\text{Grams } d\text{-xylose supplied}}$.

TABLE 4

The oxidation of d-ribose by Pseudomonas

CULTURE	AGE AT HARVEST	*d*-RIBOSE CONSUMED*	*d*-RIBONIC ACID		
			Produced†	Yield based on *d*-ribose	
				Consumed‡ (theoret.)	Supplied§ (weight)
	days	*g/culture*	*g/culture*	*per cent*	*per cent*
P. fluorescens 6	5	5.7	4.11‖	65.8	70.0
P. ovalis 8	5	5.7	1.15	18.3	19.6
P. graveolens 14	3	5.8	2.37‖	37.0	40.4
P. mildenbergii 21	5	5.6	1.01	18.2	18.7
P. vendrelli 23	7	5.6	4.56‖	75.5	77.8
P. pavonacea 24	9	0.4			
P. fragi 25	5	5.8	3.12‖	49.2	54.4
P. mephitica 75	9	1.8			
P. putrifaciens 76	9	3.5	0.78	20.2	13.3
P. synxantha 79	5	5.8	2.57‖	40.5	43.9

* Five and nine-tenths g *d*-ribose supplied per culture.

† Calculated from data on calcium in solution.

‡ One g *d*-ribose can yield 1.107 g *d*-ribonic acid.

§ $\frac{\text{Grams } d\text{-ribonic acid produced}}{\text{Grams } d\text{-ribose supplied}}$.

‖ Benzimidazole derivatives prepared and identity established by X-ray.

of the compounds was further established by comparison of X-ray diffraction patterns.

DISCUSSION

Many strains among the various species of *Pseudomonas* are capable of oxidizing pentoses to the corresponding pentonic acids. Similar oxidations have been known for many years anong the acetic acid bacteria. Bertrand (1898*a*, 1898*b*) demonstrated the production of xylonic acid from xylose and arabonic acid from arabinose by the sorbose bacterium (*Acetobacter xylinum*). Further similarities between bacteria of the genera *Acetobacter* and *Pseudomonas* lie in the facts that glucose is oxidized to gluconic acid by many species of both genera, and that gluconic acid is further oxidized by bacteria of each genus to 2-oxogluconic acid although, among the acetic acid bacteria, 2-oxogluconic acid is accompanied by the 5-oxogluconic acid, which is generally the major metabolic product (Lockwood, Tabenkin, and Ward, 1941; Prescott and Dunn, 1940). Further study of the oxidative capacity of bacteria of the genus *Pseudomonas* will doubtless reveal as interesting and valuable a series of biochemical conversions as are now known among bacteria of the genus *Acetobacter*.

It is noteworthy that cultures of *P. fragi*, *P. synxantha*, and *P. vendrelli* oxidized both the *d*- and *l*-enantiomorphs of arabinose.

No effort has been made to find the conditions under which the maximal yields of pentonic acids may be obtained. It is probable, however, that bacterial oxidation with *Pseudomonas* may prove a convenient method for the preparation of these acids from the corresponding sugars.

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SUMMARY

Pseudomonas fragi, *P. graveolens*, *P. synxantha*, and *P. vendrelli* oxidized *d*-arabinose to *d*-arabonic acid when grown in aerated corn steep liquor solutions in the presence of $CaCO_3$.

Pseudomonas fluorescens, *P. fragi*, *P. mildenbergii*, *P. putida*, *P. synxantha*, and *P. vendrelli* oxidized *l*-arabinose to *l*-arabonic acid when grown in aerated corn steep liquor solutions in the presence of $CaCO_3$.

Pseudomonas fluorescens, *P. fragi*, *P. graveolens*, *P. mildenbergii*, *P. ovalis*, and *P. putida* oxidized *d*-xylose to *d*-xylonic acid when grown in aerated corn steep liquor solutions in the presence of $CaCO_3$.

Pseudomonas fluorescens, *P. fragi*, *P. graveolens*, *P. mildenbergii*, *P. ovalis*, *P. putrifaciens*, *P. synxantha*, and *P. vendrelli* oxidized *d*-ribose to *d*-ribonic acid when grown in aerated corn steep liquor solutions in the presence of $CaCO_3$.

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ANTIBIOTIC ACTIVITY OF SOME CRUDE PLANT JUICES

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It has been repeatedly demonstrated that some plant juices contain antibacterial and antifungal substances (Irving, Fontaine, and Doolittle, 1945; Osborn, 1943; Lucas and Lewis, 1944; Sanders, Weatherwax, and McClung, 1945; Huddleson, DuFrain, Barrons, and Giefel, 1944; Cavallito, Bailey, and Kirchner, 1945; Pederson and Fisher, 1944). Many of these studies have been directed toward uncovering compounds active against human pathogenic organisms. Recently, however, Irving, Fontaine, and Doolittle (1945) announced the discovery of a substance in the juice of the tomato that strongly inhibited the growth of *Fusarium oxysporum* f. *lycopersici*, the organism causing *Fusarium* wilt of tomatoes. This substance was named "lycopersicin," which was later changed to "tomatin." These investigators pointed out that the resistance of some plant varieties to phytopathogens might conceivably be related to the presence of more or less specific antibiotic substances in the juices of these plants.

The work in these laboratories was undertaken with a view to testing the validity of this postulate, and accordingly seeds of resistant and nonresistant varieties of common garden plants were secured. From these seeds plants were grown in the greenhouse, and juice was expressed from the leaves and stems by means of a screw press. The juices were centrifuged, and the supernatants were immediately sterilized by passage through a Seitz filter and stored in the refrigerator in sterile bottles. Approximately 75 ml of juice were sterilized in this manner, and the first filtrate to come through was not discarded, as is often done. The juices were tested within a few days against various phytopathogens, using the cylinder plate method described by Irving, Fontaine, and Doolittle (1945). The four pathogenic fusaria were grown on plates containing Czapek glucose agar, as suggested by these workers. The phytopathogenic bacteria were grown on Difco nutrient agar and the tests run in the same manner. These crude undiluted extracts were also assayed against *Staphylococcus aureus*, *Eberthella typhosa*, *Salmonella paratyphi*, and *Escherichia coli*. No attempt was made to make any of the assays quantitative. The test plates were prepared as follows: (1) A 24-hour culture of the various fusaria in "hormone" beef infusion broth containing 2 per cent glucose was shaken with glass beads to break up the mycelium; 1-ml portions of these suspensions were pipetted into sterile petri plates, and 10 ml of a modified Czapek glucose agar (Irving, Fontaine, and Doolittle, 1945) were added. (2) The plant and animal pathogenic bacteria were grown for 24 hours in nutrient broth (Difco), and 1-ml portions of these suspensions were plated in 1.5 per cent nutrient agar (Difco). Penicylinders were affixed to the surface of

TABLE 1

Effect of plant juices on human bacterial pathogens

PLANT	DESCRIPTION	HUMAN PATHOGENS			
		Staphylococcus aureus FDA 209	*Eberthella typhosa* FDA Hopkins	*Salmonella paratyphi* A	*Escherichia coli*
Bean, Great Northern	Resistant to halo blight caused by *Phytomonas medicaginis* var. *phaseolicola*	0	0	10	+
Bean, Hidatsa Red	Resistant to halo blight caused by *Phytomonas medicaginis* var. *phaseolicola*	0	15	10	+
Bean, White Kidney	Susceptible to halo blight	0	15	8	+
Corn, Iowa Inbred, 4473	Resistant to bacterial wilt caused by *Bacillus stewartii*	7	0	0	—
Corn, Iowa Inbred, 4528	Resistant to bacterial wilt caused by *Bacillus stewartii*	7	25	15	20
Corn, Iowa Inbred G.B. 134	Susceptible to bacterial wilt	0	20	22	20
Corn, Iowa Inbred G.B. 797	Susceptible to bacterial wilt	—	—	22	—
Cabbage, Wisconsin Jersey Queen	Resistant to cabbage yellows caused by *F. oxysporum* f. *conglutinans*	0	10	0	—
Cabbage, Wisconsin All Head	Resistant to cabbage yellows caused by *F. oxysporum* f. *conglutinans*	0	0	0	—
Cabbage, Penn State Ballhead	Susceptible to cabbage yellows	0	0	0	—
Cabbage, Danish Red	Susceptible to cabbage yellows	—	0	0	+
Mustard, Wild		0	0	0	0
Cucumber, Ohio 31	Resistant to bacterial wilt caused by *Erwinia tracheiphila*	0	0	0	—
Cucumber (Hybrid) W-4 Self	Resistant to bacterial wilt caused by *Erwinia tracheiphila*	0	0	0	—
Cucumber, Tokyo Long Green	Susceptible to bacterial wilt	0	0	0	—
Tomato, Pan-American	Resistant to fusarium wilt caused by *Fusarium oxysporum* f. *lycopersici*	0	0	0	—

TABLE 1—*Concluded*

PLANT	DESCRIPTION	HUMAN PATHOGENS			
		Staphylococcus aureus FDA 209	*Eberthella typhosa* FDA Hopkins	*Salmonella paratyphi* A	*Escherichia coli*
Tomato, Break O'Day	Resistant to fusarium wilt caused by *Fusarium oxysporum* f. *lycopersici*	0	0	15	–
Tomato, Bonny Best	Susceptible to fusarium wilt	8	0	0	–
Cauliflower W. S. 300	Resistant to black rot caused by *Phytomonas campestris*	0	10	0	0
Cauliflower, Improved Holland Erfurt	Susceptible to black rot	0	10	0	0

+ = growth stimulation.
0 = no inhibition.
– = no assay.
Numerals indicate size of inhibition zone in millimeters.

these plates immediately after hardening of the agar, and filled with the juices to be tested. Preliminary incubation, for a period of 18 hours, of the plates containing the four species of fusaria was necessary before placing and filling the penicylinders, but was not necessary in the case of the plates containing the bacterial species. The plates containing bacteria parasitic for plants were incubated at room temperature (18 to 25 C), and those containing zoopathogens were incubated at 37 C. The juices were adjusted to the optimum pH before use. The diameters of the inhibition zones were measured and recorded in millimeters.

In some instances there was no visible zone, but removal of the penicylinder revealed a clear area. When this occurred, the zone was listed as 7 mm.

The results obtained when these juices were tested against some human bacterial pathogens are shown in table 1.

The same juices were all tested against seven phytopathogenic bacteria, *Phytomonas medicaginis* var. *phaseolicola, Bacillus stewartii, Actinomyces scabies, Erwinia amylovora, Erwinia carotovora, Phytomonas campestris,* and *Phytomonas solanacearum,* with the following results: The juices of all three bean varieties were stimulatory to both *E. carotovora* and *E. amylovora.* Corn, Iowa Inbred no. 4528 (*B. stewartii*—18 mm; *E. carotovora*—10 mm); cabbage, Wisconsin Jersey Queen (*P. campestris*—8 mm); wild mustard (*P. campestris*—10 mm); cucumber, Ohio 31 (*E. carotovora*—10 mm). Juices of the three tomato varieties were all inactive against these oganisms.

When the same juices were used against four phytopathogenic fusaria, the following positive results were obtained: Corn, Iowa Inbred no. 4473 (*F. oxysporum* f. *niveum,* Snyder & Hansen—7 mm; *F. oxysporum* f. *melonis,* Snyder & Hansen—7 mm); corn, Iowa Inbred no. 4528 (*F. oxysporum* f. *melonis*—7 mm);

cabbage, Penn State Ballhead (*F. oxysporum* f. *conglutinans*, Snyder & Hansen—+); tomato, Pan-American (*F. oxysporum* f. *conglutinans*—16 mm; *F. oxysporum* f. *lycopersici*, Snyder & Hansen—7 mm; *F. oxysporum* f. *niveum*—+); tomato, Break O'Day (*F. oxysporum* f. *conglutinans*—7 mm; *F. oxysporum* f. *lycopersici*—7 mm; *F. oxysporum* f. *niveum*—+; *F. oxysporum* f. *melonis*—7 mm); tomato, Bonny Best (*F. oxysporum* f. *conglutinans*—16 mm; *F. oxysporum* f. *niveum*—+); cauliflower, W. S. 300 (*F. oxysporum* f. *conglutinans*—+). All other juices were inactive.

Although some antibiotic substances have been shown to be active against plant pathogenic organisms (Brown and Boyle, 1945; Waksman, Bugie, and Reilly, 1944), it appears that such compounds are not widespread in the juices of common garden plants. Of the plants tested, only corn, cucumber, wild mustard, and cabbage demonstrated any activity against the bacterial phytopathogens, whereas the juice of corn and tomatoes was detrimental to the growth of the plant pathogenic fusaria. A rather pronounced variation in susceptibility of the four fusaria to these juices is demonstrated. Also, it is interesting that although the different tomato varieties yield juices more active against the fusaria than do the corn varieties, the latter are inhibitory to the bacterial phytopathogens which are completely resistant to the action of the tomato plant juices.

The data indicate that these juices are much more active against animal pathogens than against those causing diseases of plants. Also, the inhibition seems to be more pronounced in the case of the three gram-negative organisms than against the gram-positive organism, *Staphylococcus aureus*. Beans, corn, cabbage, cauliflower, and tomatoes are active, with the juice of corn being the most generally effective.

The many cases of growth stimulation may be due to specific stimulatory substances, or they may perhaps be examples of border-line inhibition. Further concentration and purification of these juices should yield information on this point.

It is obvious that no clear distinction may be drawn between the activities of the juices of resistant and nonresistant varieties, except perhaps in the case of the action of corn juices against the bacterial phytopathogens. However, the fact that these differences have not been demonstrated does not mean that they are nonexistent. Indeed, it is probable that in at least some of the cases our methods were not accurate enough to be useful in bringing out differences which are probably quantitative, rather than qualitative, in nature.

Work is continuing at this laboratory on the purification and characterization of the most promising of these factors.

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CHARACTERISTICS OF LACTIC ACID BACTERIA FROM COMMERCIAL CUCUMBER FERMENTATIONS[1]

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In commercial practice cucumbers are brined in wooden vats ranging in capacity from about 100 to 1,200 bushels. The vats, after being filled with green cucumbers, are fitted with false heads, and salt brine of a given concentration is added to a level a few inches above the head. Next, dry salt is added on the false head of the vat to maintain the initial concentration, which otherwise would become diluted by the water from the cucumbers. The initial brine strength used ordinarily ranges from about 8 to 10 per cent, depending upon the individual plant concerned. In most instances the brine concentration is gradually raised so that a holding strength of about 16 to 18 per cent is reached after about 6 weeks. Under these conditions an active acid fermentation resulting from the growth of salt-tolerant, acid-forming bacteria usually begins within a day or so after the cucumbers are brined and may continue for about 6 weeks. The preserving effect of the brine is due chiefly to the combined action of the salt and the developed acidity.

The role of the acid-forming bacteria in commercial cucumber fermentations, from the standpoint of populations occurring at various brine strengths and their relation to the principal chemical changes taking place in the brines, is probably better understood than the identity of the organisms concerned. The present study was undertaken in an effort to obtain more specific information on the nature of the predominating lactic acid bacteria involved during the fermentation of salt-stock cucumbers, under conditions typical of the industry.

EXPERIMENTAL

Particular emphasis in the current investigation has been placed on 36 isolates obtained during the 1938 season. These were isolated from the active phase of the acid fermentation of 85-bushel lots of cucumbers brined at about 5, 8, and 10.5 per cent salt concentration.[2] Information concerning the source of these cultures and other pertinent facts on brining treatment and fermentation activity are given in table 1. In addition to these cultures, 13 random isolates from six 720-bushel lots of fermenting cucumbers at various stages of activity were also studied. These lots received essentially the 10.5 per cent brine treatment outlined in table 1 for vat 14. Details concerning the origin of the cultures from these fermentations are presented in table 2.

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[2] In the cucumber pickling industry, brine salinity is usually recorded in terms of degrees salometer (° sal.) as measured by a hydrometer calibrated in percentage of saturation with respect to sodium chloride (0 to 100° sal.). In the brining treatments described herein, commercial practices were followed throughout; however, for the convenience of the reader, degrees salometer have been converted to the approximate equivalent in percentage of salt by weight (e.g., 20, 30, and 40° sal. brines would approximate 5, 8, and 10.5 per cent salt brines).

The isolates studied were considered representative of a larger group of cultures collected over a period of several seasons from actively fermenting commercial cucumber brines. Fermentations occurred for the most part during the month of July, with brine temperatures within the range of 27 to 30 C. All vats were located outside and were unsheltered. The investigations were conducted at two large pickling plants located in eastern North Carolina.

TABLE 1

Origin of cultures isolated in sequence from cucumber fermentations (85-bushel lots) receiving 5, 8, and 10.5 per cent brine treatments

LOT NUMBER (1938)	BRINING TREATMENT DESIGNATION AND INITIAL CONCENTRATION THE 1ST WEEK*		RATE OF INCREASE OF BRINE CONCENTRATION PER WEEK		NUMBER OF ISOLATES	FERMENTATION PERIOD COVERED BY ISOLATIONS	MEAN PLATE COUNT OF ACID-FORMING BACTERIA DURING ISOLATION PERIOD	MAXIMUM BRINE ACIDITY AS LACTIC	MINIMUM BRINE pH
	per cent	*°sal.*	*per cent*	*°sal.*		*days*	*millions per ml*	*per cent*	
Vat 8	5.0	20	2.5	10	21	3rd to 13th†	73.1	0.78 (10th day)	3.31
Vat 13	8.0	30	2.0	8	6	3rd to 13th‡	66.2	0.60 (12th day)	3.30
Vat 14	10.5	40	1.25	5	9	5th to 13th§	4.2	0.41 (15th day)	3.60

* In the three brining treatments shown (5.0, 8.0, and 10.5 per cent), the initial brine concentration was maintained for the first week and then increased as indicated (column 3) until 15 per cent was reached. All vats were filled June 27, 1938.

† Two cultures isolated per day during the period, except one isolation on the 6th day.

‡ One culture each was isolated on the 3rd, 10th, 11th, and 12th days; two cultures on the 13th.

§ Two cultures each were isolated on the 5th, 7th, 11th, and 13th days; one culture on the 9th.

All isolates were obtained by picking representative colonies from plates of nutritive caseinate agar. This medium was used for the purpose of following the populations of acid-forming bacteria in the fermentations studied. Also, it has been found adequate for the isolation of both gas-forming and non-gas-forming types of lactic acid bacteria in certain pickle products, particularly low-salt-content dills and improperly pasteurized fresh cucumber pickles, in which both types are commonly found.

The cultures were studied during the spring of 1939, and the criteria for classification included morphological observations and determination of cultural characteristics on various liquid and solid media (i.e., milk with indicator, potato, cucumber-juice agar, cucumber-juice broth, nutrient broth, nutrient agar, nutritive caseinate agar with indicator, glucose tryptone agar with indicator, glucose agar, glucose broth, and gelatin glucose stab). Other tests included relation to oxygen, action on casein and nitrates, indole production, optimum growth temperature, action on carbon compounds, relation to salt, thermal death temperature, and carbon dioxide production. The methods employed were essentially those suggested by the work of Pederson (1930, 1936, 1938).

TABLE 2

*Origin of random cultures isolated from six cucumber fermentations (720-bushel lots) receiving 10.5 per cent brine treatment**

LOT NUMBER (AND DATE FILLED—1936)	NUMBER OF ISOLATES AND FERMENTATION AGE WHEN ISOLATED		BRINE CONCENTRATION AT TIME OF ISOLATIONS		PLATE COUNT OF ACID-FORMING BACTERIA AT TIME OF ISOLATIONS	BRINE ACIDITY AS LACTIC		BRINE pH AT TIME OF	
						At time of isolations	Maximum developed	Isolations	Minimum
	number	*day*	*per cent*	*°sal.*	*millions per ml*	*per cent*	*per cent*		
Vat 124 (7-1)	2	11th	11.4	43	3.1	0.44	0.45 (16th day)	3.60	3.60
Vat 125 (7-6)	2	5th	10.8	41	12.8	0.21	0.44 (12th day)	3.93	3.57
Vat 126 (7-1)	3	12th	11.4	43	0.2	0.50	0.54 (18th day)	3.50	3.50
Vat 3 (6-29)	1	9th	12.4	47	0.1	0.58	0.64 (15th day)	3.59	3.58
Vat 21 (7-8)	1	4th	11.1	42	0.8	0.16	0.45 (17th day)	5.18	3.67
Vat 17 (7-7)	1	4th	11.4	43	19.0	0.13	0.38 (18th day)	4.96	3.67
	2	7th	12.1	46	3.0	0.30		3.85	
	1	8th	12.4	47	1.7	0.33		3.77	

* This brining treatment was essentially the same as for vat 14 shown in table 1.

Other details, such as those concerning the preparation of the various cultural media employed, preparation of the carbon compounds used in the fermentation tests, determination of titratable acidity and pH, and other tests incident to the study have recently been described by Etchells, Fabian, and Jones (1945) and Etchells and Jones (1946). The actual species allocation was on the basis of the characteristics set forth by Pederson (1936) and by Bergey *et al.* (1939).

The results with respect to morphological, cultural, and certain of the biochemical characteristics for the cultures will not be given in detail, since they were entirely typical of those described for the *Lactobacillus* species identified. Summarized results for additional tests, such as action on carbon compounds, relation to salt, thermal death temperature, and carbon dioxide production are presented below.

Action on carbon compounds. Definite acid fermentation is shown by the majority of strains on *l*-arabinose, glucose, *d*-galactose, lactose, fructose, maltose, *d*-mannose, raffinose, salicin, and sucrose. In general, the action on sucrose and raffinose varies more than with the other compounds in this group. Less active acid fermentation is obtained with most strains on dextrin, glycerol, mannitol, *d*-sorbitol, and *l*-xylose, as indicated by both the number of nonfermenters and the smaller amounts of acid produced. The compounds inulin, rhamnose, starch, and melezitose are fermented either not at all or, at best, only to a rather limited degree.

Relation to salt. In cucumber fermentations little or no growth is found at 15 per cent salt concentration or above. At brine concentrations below 15 per cent there is an inverse relationship between populations found and the salt concentration employed. Correspondingly lower populations and brine acidity are observed as the salt content of the brine is increased up to the inhibiting range (15 per cent). Laboratory tests on the salt tolerance of cultures may not provide a reliable index to their behavior toward salt under natural conditions. Cultures isolated from actively fermenting brines at 10 to 12 per cent salt concentration may not show growth in liquid media plus salt much above one-half the original isolation concentration.

Thermal death temperature. This is about 65 to 70 C for 15 minutes. Some strains may survive 60 C for 15 minutes, but usually they are killed by exposure to 65 C for the same time interval. Cultures from cucumber fermentations have not been observed to withstand 70 C for 15 minutes.

Carbon dioxide production. Strains not considered gas-producing in the sense that the term applies to the four gas-producing species of the *Lactobacillus* genus. The mean carbon dioxide production for 49 strains tested (with Eldredge tubes) in the present study was 5.4 per cent. About 20 per cent is the usually accepted range for members of the gas-producing species (Hucker and Pederson, 1930; Pederson, 1931, 1939).

The results with respect to the characteristics of the cultures investigated assure identification of the 36 strains from 5, 8, and 10.5 per cent brines among the non-gas-producing species of the genus *Lactobacillus*. Furthermore, it appears certain that the characteristics are distinctly more typical of those described for *Lactobacillus plantarum* (Orla-Jensen) Bergey *et al.* than for those of the remaining non-gas-producing species listed (Bergey *et al.*, 1939). On the basis of results from similar identification studies (particularly with respect to carbohydrate fermentations and carbon dioxide tests), the 13 random cultures, from six other commercial fermentations, are likewise considered as belonging to the species *Lactobacillus plantarum.*

DISCUSSION

The results of this investigation indicate that *L. plantarum* was chiefly responsible for the brine acidity of the fermented cucumbers and that other common types of lactic acid bacteria, such as species of *Leuconostoc* or the gas-producing species of *Lactobacillus*, did not contribute materially to acid formation in the fermentations studied. The latter conclusion is based on the fact that no members of these genera were isolated either from the brines that were studied at rather close intervals during the active phase of acid fermentation or from those that were sampled at random.

In considering the inactivity of the gas-producing lactobacilli (e.g., *L. brevis*) in the brines studied, it appears likely that brining procedures using salt concentrations of 5 to 10 per cent exert a more inhibitive influence on the population development of these types than on that of the non-gas-producers (e.g., *L. plantarum*). The work of Vahlteich, Haurand, and Perry (1935) lends support to this view. They concluded that acid formation in two commercial cucumber fermentations at 10 per cent brine strength was due principally to *Lactobacillus cucumeris* (syn. *L. plantarum*); no isolates of the gas-producing lactobacilli were obtained. Further evidence of the effect of salt on the gas-producing types is found in the work on olive fermentations by Vaughn and coworkers (1943). They found these species active during the fermentation of the Sevillano variety of olives but not with the Manzanillo variety. They attributed this difference in fermentation behavior to the fact that the latter variety, in commercial practice, is brined at twice the salt concentration used for the former.

The possible influence of brine temperature and brine acidity should also be considered. The temperature requirements for certain members of both the non-gas-producing and gas-producing groups are rather similar (optimum for *L. plantarum* and *L. brevis*, 30 to 35 C). Hence, it scarcely seems plausible that the brine temperatures (27 to 30 C) encountered during the cucumber fermentations described herein could be offered as an important factor in the apparent inactivity of the gas-producing species, particularly to the extent attributed to the effect of salt. Also, the amount of brine acidity produced by the acid-forming bacteria identified as *L. plantarum* would not be considered sufficient to preclude subsequent development of gas-producing types.

The foregoing discussion has dealt principally with the influence of salt concentration on the gas-forming types of lactobacilli. In considering the failure to obtain isolates of the *Leuconostoc* genus in the cucumber fermentations studied, the influence of brine temperature as well as salt concentration must be recognized. According to the work of Pederson (1930, 1931) on sauerkraut, brine temperatures of about 30 C would not encourage rapid development of members of the *Leuconostoc* genus as compared with that of acid formers such as *L. plantarum*, even at salt concentrations well within the growth limits of both groups of organisms. As a consequence, *L. plantarum* might be expected to dominate the acid fermentations observed in the present study, both on the basis of more vigorous growth in brines at a temperature of about 30 C and as the result of higher salt tolerance as compared with the *Leuconostoc*. Although two strains of *Leuconostoc* were isolated in the studies previously referred to by Vahlteich *et al.*, these organisms were considered of minor importance in the acid fermentation.

Under certain conditions, particularly when cucumbers were put down at brine temperatures of about 20 C, an occasional fermentation has been observed by the authors to develop an abnormal viscid or syrupy type of brine. This condition, in addition to being associated with cool weather during the filling and brining operation, is also usually limited to brine treatments in the range of 5 per cent initial strength. Observations on the predominating lactic acid bacteria present in the brine have shown them to be gram-positive, gas-producing cells, ranging from short rods to spheres in shape, producing slime on sucrose media, and capable of producing more than 0.8 per cent acid (calc. as lactic) in cucumber-juice broth. In the absence of detailed taxonomic studies, these observations strongly suggest that the abnormal brine consistency occasionally observed is associated with a predominating flora of members of the *Leuconostoc* group. Such an explanation would be in keeping with the lower temperature requirements and lower salt tolerance of this group.

The possibility of influencing the character of the acid fermentation by the type of plant material employed is worthy of mention. Work in this direction would indicate that marked microfloral changes are not to be expected, provided different materials are brined or salted at similar concentrations and under similar conditions, and also contain sufficient amounts of readily fermentable carbohydrate. This is borne out in the fermentation of sauerkraut as described by Pederson (1930) and the fermentation of Sevillano olives reported by Vaughn *et al.* (1943). In these two studies the microfloral changes were remarkably similar, both with respect to the predominating types of acid-forming bacteria found and the sequence in which they occurred. Both groups of workers reported that the developed acidity resulted chiefly from three groups of acid-forming bacteria; i.e., gas-producing cocci of the *Leuconostoc* genus and non-gas-producing and gas-producing bacilli of the *Lactobacillus* genus (*L. plantarum* and *L. brevis*,[3] respectively). These occurred in the order named. In both cases, the fermentations involving these dissimilar types of material took place at relatively low salt concentrations (about 2.5 per cent for sauerkraut, and about 3 to 4 per cent for olives). In recent studies by the authors, it was observed that during the fermentation of a number of different types of vegetables (e.g., corn, peas, green beans, and okra) the principal influence on the acid-forming bacteria was exerted by the salt concentration used in the preservation treatment rather than by the type of material studied.

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[3] Referred to by Pederson (1930) as *L. pentoaceticus*. In accord with later work by Pederson (1938) this species is now considered identical with *L. brevis* (Bergey *et al.*, 1939).

SUMMARY

Identification studies on 49 cultures of lactic acid bacteria occurring during the acid fermentation of salt-stock cucumbers, under conditions typical of the industry, are reported.

Thirty-six of the cultures were isolated during the active phase of acid formation from fermentations maintained at about 5, 8, and 10.5 per cent salt concentration for 1 week, after which the brine strength was gradually increased. These isolates gave characteristics typical of those described for *Lactobacillus plantarum* (Orla-Jensen) Bergey *et al.* and were allocated to this species.

The remaining 13 cultures were isolated at random from six cucumber fermentations at brine concentrations ranging from about 11 to 12.5 per cent salt. These isolates were also considered to belong to the species *Lactobacillus plantarum*.

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PARTIAL ANTIBIOTIC SPECTRUM OF TOMATIN,[1] AN ANTIBIOTIC AGENT FROM THE TOMATO PLANT[2]

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The clinical attractiveness of antibiotic agents, as typified by penicillin and streptomycin, lies in their ability to effect striking and specific bacteriostatic action *in vivo* without the simultaneous production of severe toxic symptoms. However, since penicillin and streptomycin are limited in their usefulness because of their ineffectiveness against certain important groups of pathogenic organisms, the search for new antibiotic agents continues in the hope that additional substances having sufficiently low toxicity will be found whose high antibiotic activity against the penicillin- and streptomycin-resistant organisms will permit their therapeutic use in the conquest of the diseases caused by these pathogens.

Although the fungi, including *Actinomyces* and related forms, and bacteria have been the most fruitful sources of antibiotic agents, antibiotic activity has also been attributed to the juices of certain green plants. Many plant families have been examined for antibiotic activity (Osborn, 1943; Huddleson *et al.*, 1944; Lucas and Lewis, 1944; Seegal and Holden, 1945), and several plant constituents that possess antibiotic activity have been isolated in crystalline form. Among these antibiotic agents are a substance from garlic (*Allium sativum*) that has been tentatively identified as the sulfoxide of diallyl disulfide (Cavallito, Buck, and Suter, 1945); a substance from common burdock (*Arctium minus*) that has not been identified but which appears to be a lactone having the empirical formula $C_{15}H_{20}O_5$ (Cavallito, Bailey, and Kirchner, 1945); and a substance designated "crepin" from *Crepis taraxacifolia* that has the empirical formula $C_{14}H_{16}O_4$ (Heatley, 1944).

It is the purpose of this paper to describe some of the antibiotic properties of what is believed to be a new antibiotic agent from a plant source. This substance occurs in the tomato plant and has been designated "tomatin." Tomatin has not yet been crystallized, but preparations of sufficient potency have been obtained to warrant a preliminary investigation of its antibiotic spectrum. Because of the probable impurity of the tomatin preparation used in the present investigation, the data to be presented have only qualitative or, at best, semiquantitative significance. Investigation of the activity of tomatin with respect to the organisms employed in the present work will be repeated when purer tomatin preparations are isolated, and experimentation will be extended to include other pathogens.

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[1] In a recent publication (Irving, Fontaine, and Doolittle, 1945) this substance was referred to as "lycopersicin." Inasmuch as it has since been learned that the term "lycopersicin" was once used (Duggar, 1913) as a synonym for "lycopene," the red pigment of the tomato, the designation of the antibiotic agent has been changed to "tomatin" to avoid possible confusion.

[2] Presented before the District of Columbia Section, Society for Experimental Biology and Medicine, December 6, 1945.

EXPERIMENTAL

Preparation of tomatin. Details of the procedure for the isolation of potent tomatin preparations from the tomato plant and a discussion of the chemical and physical properties of tomatin will be presented elsewhere. The tomatin preparation used in the present investigation was prepared in the following manner: The mechanically expressed juice of thoroughly washed, mature, Red Currant tomato plants (*Lycopersicon pimpinellifolium*) was autoclaved, and the clear extract obtained by centrifuging was concentrated to dryness *in vacuo* at 60 C. The extract obtained by thorough extraction of the residue with absolute methanol was concentrated to dryness *in vacuo*, and an aqueous solution of the residue was sterilized and stored in the cold for use in these experiments. This solution (pH 4.0) contained approximately 70 tomatin units per ml when assayed by the procedure previously described (Irving, Fontaine, and Doolittle, 1945).

Procedure. Sterile, 90-mm petri dishes, containing 20 ml of solidified nutrient agar,[3] were warmed to 45 C and flooded with 3 ml of a suspension of bacterial cells or fungus spores in the same medium. The inoculum was prepared by adding to 10 ml of melted medium (cooled to 45 C) 1 ml of a suspension obtained by washing the surface of a vigorously growing agar slant culture of the organism with 5 ml of sterile water. Five porcelain cylinders (8 mm by 10 mm high) were dropped on the solidified, inoculated surface of the plate; suitable dilutions of the sterile tomatin stock solution were pipetted into three of the cylinders; and the plates were incubated at 28 or 40 C until growth of the organism was sufficiently advanced to permit accurate measurement of the inhibition zones produced. In nearly all instances dilute solutions of penicillin ("penicillin-sodium," Chas. Pfizer and Co., Inc.) were placed in the two remaining cylinders on each of the plates for comparison. The penicillin solutions were standardized by assay against *Staphylococcus aureus* (Schmidt and Moyer, 1944).

Results. The effectiveness of tomatin and of penicillin in inhibiting cultures of four bacteria and ten fungi is shown in table 1. To facilitate comparison, only the results for solutions containing 5 units of tomatin and 4 units of penicillin per ml are given in the table. Experiments have also been conducted at various times with tomatin concentrations of 1 and 10 units per ml and penicillin concentrations of 2.5 and 20 units per ml. In all instances the diameters of the inhibition zones produced by lower or higher concentrations of tomatin corresponded closely with the values that would be expected on the basis of the figures given in the table. Since the tomatin solutions used had pH values of approximately 4.0, it was desirable to test the effect of weak acid solutions alone on each of the organisms. None of the organisms listed in table 1 was inhibited when a solution containing 0.5 g KCl, 0.5 g Mg SO_4, and 1.0 g KH_2PO_4 and having a pH of 3.5 was used in place of tomatin in a cylinder of the test plate.

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[3] Medium: bacto yeast extract dehydrated, 5 g; bacto peptone, 5 g; glucose anhydrous Squibb, 2.5 g; bacto agar, 15 g; distilled water to 1 liter; pH 6.4.

Typical cylinder plates, which illustrate the effect of tomatin on *Trichophyton mentagrophytes* and the three *Fusarium* species, are shown in figure 1. The effectiveness of tomatin, when added to the culture medium, in inhibiting several organisms is illustrated in figure 2.

TABLE 1

Antibiotic effects of tomatin and penicillin on certain bacteria and fungi

ORGANISM*	INCUBATION PERIOD	DIAMETER OF INHIBITION ZONE, MILLIMETERS	
		Tomatin	Penicillin
	hr	*5 u/ml*	*4 u/ml*
Staphylococcus aureus (NRRL B-313)†	18	17	28
Bacillus subtilis (NRRL 558)†	18	22	29
Escherichia coli (NRRL B-210)†	18	0	0
Phytomonas solanacearum	20	16	0
Penicillium notatum (NRRL 1249B21)	48	0	0
Aspergillus clavatus (ATCC 1007)	19	23	
Aspergillus clavatus (ATCC 9192, Waksman 129)	19	21	
Fusarium oxysporum f. *lycopersici* (R-5-6)	23	23	0
Fusarium oxysporum f. *pisi* (SPD 340)	48	24	0
Fusarium oxysporum f. *conglutinans* (SPD 341)	42	32	0
Candida albicans (ATCC 2091)	23	20	0
Trichophyton mentagrophytes (ATCC 9533)	89	36	0
Epidermophyton floccosum (ATCC 9646)	120	34‡	0
Microsporum audouini (ATCC 9082)	43	§	

* NRRL, Northern Regional Research Laboratory; ATCC, American Type Culture Collection; R-5-6, highly virulent strain from collection of Dr. F. L. Wellman (Wellman, 1942); SPD, Doolittle collection, originally obtained from the collection of Dr. J. C. Walker. The culture of *P. solanacearum* was a fresh isolate taken from a severely diseased tomato plant.

† Incubated at 40 C; all others incubated at 28 C.

‡ Inhibition zone produced by 1 unit tomatin per ml.

§ Growth too slow for satisfactory application of cylinder-plate technique. However, this organism is strongly inhibited when tomatin is added to the culture medium (see figure 2).

As was to be expected, penicillin was effective only against the two gram-positive organisms, *Staphylococcus aureus* and *Bacillus subtilis*. Like penicillin, tomatin was found to be effective against these two organisms and ineffective against gram-negative *Escherichia coli*. However, unlike penicillin, tomatin was found to be effective against the gram-negative, bacterial plant-wilt pathogen *Phytomonas solanacearum*. Tomatin appeared to be without significant action upon the fungus *Penicillium notatum*, but it exhibited marked fungistatic activity toward two strains of *Aspergillus clavatus*. On the *Aspergillus clavatus* plates, after 19 hours of incubation, the inhibition zones were perfectly clear and devoid of all growth, indicating the possible fungicidal action of tomatin upon this organism. After 47 hours of incubation there appeared wide halos of appressed growth surrounding the clear zones.

The most striking antibiotic effects of tomatin were observed, however, in the case of the three plant-wilt pathogens, *Fusarium oxysporum* f. *lycopersici* (tomato wilt), *F. oxysporum* f. *pisi* (pea wilt), and *F. oxysporum* f. *conglutinans* (cabbage yellows), and in the human dermatophytes, *Candida albicans*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, and *Microsporum audouini*. It will be recalled that all of these fungi are representatives of the so-called *Fungi Imperfecti*, whose "perfect" or sexual forms are not known. Most of the fungi parasitic in man are in this group (Zinsser and Bayne-Jones, 1934). Infections by these fungi may occur in various parts of the body and include, among others, such established clinical entities as favus, ringworm, eczema, thrush, and sprue. Many of these and similar infections are not always serious, but some are wide-

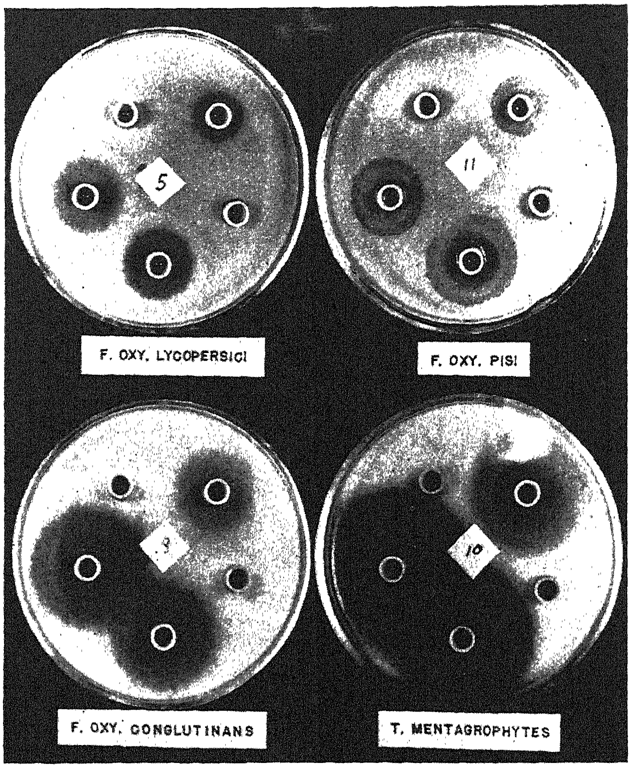

FIG. 1. EFFECT OF TOMATIN AND OF PENICILLIN ON SEVERAL FUNGI

Reading clockwise from bottom, the cylinders on plates 5, 8, and 10 contain tomatin 5 u per ml; tomatin, 10 u per ml; penicillin, 2.5 u per ml; tomatin, 1 u per ml; penicillin, 4 u per ml. The cylinders on plate 11 contain tomatin, 10 u per ml; tomatin, 5 u per ml; penicillin, 2.5 u per ml; tomatin, 1 u per ml; penicillin, 4 u per ml.

spread, often incapacitating, presistent, recurrent, and resistant to treatment. The marked activity of tomatin with respect to the representatives of the *Fungi Imperfecti* listed in table 1 suggest that tomatin may be effective against many other pathogenic fungi within this group. A more thorough investigation of the fungistatic and fungicidal activity of tomatin in relation to the imperfect fungi is in progress.

It is noteworthy that tomatin not only strongly inhibits a highly virulent strain of *Fusarium oxysporum* f. *lycopersici*, the organism that causes wilt in the tomato plant, but it also inhibits, to an equal or greater degree, the *Fusarium* species that cause similar wilt diseases in peas and cabbage. The role played by tomatin in the natural wilt resistance exhibited by some varieties of tomatoes and the significance of the antibiotic activity of tomatin toward other plant-pathogenic species of *Fusarium* and the bacterial wilt organism will be discussed elsewhere.

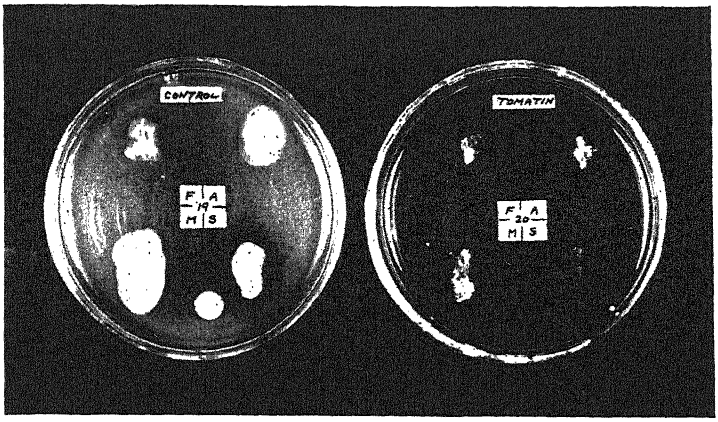

Fig. 2. Effect of Addition of Tomatin to the Medium on the Growth of Several Organisms

Plate on left contains no added tomatin; growth vigorous. Plate on right contains 1 unit of tomatin per ml of medium; growth insignificant. F, *Epidermophyton floccosum;* A, *Microsporum audouini;* M, *Trichophyton mentagrophytes;* S, *Phytomonas solanacearum*. Both plates incubated for 43 hours at 28 C.

DISCUSSION

The marked fungistatic and possibly fungicidal powers of tomatin *in vitro* encourage speculation concerning its possible therapeutic applicability in human and animal fungus infections. However, such a possibility can be entertained only if subsequent investigations, now in progress, prove tomatin to be sufficiently nontoxic to permit local or perhaps internal application in man and animals. A highly active nontoxic fungistatic agent would be of value in the parenteral or oral treatment of certain fungus infections in cases in which fungistatic agents like actinomycin and gliotoxin (Reilly, Schatz, and Waksman, 1945) are of limited usefulness because of their toxicity.

It is difficult to make a true comparison of the relative fungistatic powers of tomatin and certain other fungistatic agents, since units of measurement differ between investigators. With the best tomatin preparations so far obtained, definite fungistatic activity toward *T. mentagrophytes* can be demonstrated by the cylinder-plate method with a solution containing approximately 5 micrograms of tomatin per ml. In other words, according to one widely used method of evaluation, such a tomatin preparation would contain approximately 200,000 dilution units per gram. The published figures for the two very active fungistatic agents actinomycin and gliotoxin (Reilly, Schatz, and Waksman, 1945), both of which are presumably pure compounds, indicate that each contains approximately 5 to 6 million dilution units per gram when *T. mentagrophytes* is used as the assay organism. Tomatin, therefore, even in the impure preparations now available, approaches within a factor of approximately 25 the fungistatic activity of these crystalline antibiotic agents.

SUMMARY

Tomatin, an antibiotic agent that occurs in the tomato plant, has been shown to inhibit effectively cultures of *Staphylococcus aureus*, *Bacillus subtilis*, *Phytomonas solanacearum*, *Aspergillus clavatus*, *Fusarium oxysporum* f. *lycopersici*, *Fusarium oxysporum* f. *pisi*, *Fusarium oxysporum* f. *conglutinans*, *Candida albicans*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, and *Microsporum audouini*. It is without effect upon cultures of *Escherichia coli* and *Penicillium notatum*. These results suggest the possibility that tomatin may be useful in the treatment of certain human and animal fungus infections, provided current investigations prove tomatin to be effective *in vivo* and of sufficiently low toxicity to permit local or preferably oral or parenteral administration.

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NOTES

A RAPID METHOD OF PHASE ISOLATION IN SALMONELLA CULTURES

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The method of Gard (Z. Hyg. Infektionskrankh., **120**, 59) for isolation of temporarily suppressed phases of *Salmonella* and the modification introduced by Edwards and Bruner (Ky. Agr. Expt. Sta., Circ. 54) are effective but require two days' incubation and involve much manual work. The method to be described here permits the isolation of a desired phase in 24 hours and requires only ordinary laboratory glassware. It has been used successfully in routine typing.

A 3½-by-⅝-inch tube containing 3 ml of semisolid agar is melted and cooled to 45 to 50 C, and a 3-mm loopful of the selected antiserum is added. With a 10-ml pipette, chilled infusion broth is added to a loosely plugged 50-ml centrifuge tube until the broth in the tube reaches the 1-ml mark on the pipette as it rests on the bottom of the tube. Excess broth in the pipette is discarded, and the same pipette is used to mix the semisolid medium and antiserum. After thorough mixing, the semisolid mixture is drawn up in the pipette to the 1-ml mark, and the pipette with semisolid agar is placed in the centrifuge tube so that the tip rests on the bottom of the tube. The semisolid agar in the pipette and the infusion broth in the tube should be at the same level. The loose cotton plug from the centrifuge tube can be forced back in the tube and around the pipette. The cotton plug in the mouthpiece of the pipette is removed and the mouthpiece thoroughly flamed. A 3½-by-⅝-inch tube is inverted over the mouthpiece to exclude contaminants.

For inoculation, a special needle is used consisting of a stiff wire about 14 inches long and slightly smaller in diameter than the bore of the mouthpiece on a 10-ml pipette. A 2-inch piece of thin platinum inoculating wire is attached to the end of the stiff wire with silver solder.

To inoculate this assembly, a small amount of culture is picked up on the platinum tip of the long inoculating needle. The tube is removed from the mouthpiece of the pipette and the needle passed down the bore of the pipette. The tip of the needle should just pierce the top of the semisolid agar at the 1-ml mark. The needle is withdrawn and the tube replaced over the mouthpiece. Upon overnight incubation the organisms of the desired phase will have swarmed through the semisolid agar and passed through the small bore in the tip of the pipette, inoculating the infusion broth. The broth usually contains a fairly heavy growth within 18 to 20 hours following inoculation of the semisolid agar. The pipette containing the semisolid culture can be removed by holding a finger over the mouthpiece. The remaining broth culture may be used for agglutination tests.

THE PRODUCTION OF AN ANTIFUNGAL ANTIBIOTIC BY STREPTOMYCES GRISEUS

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Streptomycin, which is produced by strains of *Streptomyces griseus*, exhibits antibiotic activity against various gram-positive and gram-negative bacteria (Schatz *et al.*: Proc. Soc. Exptl. Biol. Med., **55**, 66) but is ineffective against the fungal pathogens of man (Robinson *et al.*: Proc. Soc. Exptl. Biol. Med., **57**, 226; Reilly *et al.*: J. Bact., **49**, 585). We have found, however, that the growth of the fungal pathogen, *Cryptococcus neoformans*, is inhibited in a 1:100 dilution of a beer of *S. griseus* containing 130 μg per ml of streptomycin but that this same organism is not inhibited by 285 μg per ml of highly purified streptomycin. This ability of the beer of *S. griseus* to inhibit the growth of *C. neoformans* sug-

TABLE 1

The highest dilution[*] of 1 gram of streptomycin or of the antifungal antibiotic from S. griseus giving complete inhibition of growth of the test organism

TEST ORGANISM[†]	ANTIFUNGAL ANTIBIOTIC FROM *S. griseus*	STREPTOMYCIN[‡]
Saccharomyces cerevisiae ATCC 918	2,500,000	<1,000
Cryptococcus neoformans	1,300,000	<1,000
Rhodotorula sp.	1,300,000	1,000
Hormodendrum pedrosoi 275	20,000	<1,000
Monosporium apiospermum	20,000	<1,000
Phialophora verrucosa	20,000	<1,000
Blastomyces dermatitidis 930	<10,000	<1,000
Candida albicans	<10,000	<1,000
Coccidioides immitis 819	<10,000	<1,000
Epidermophyton floccosum	<10,000	<1,000
Geotrichum sp.	<10,000	<1,000
Hormodendrum compactum	<10,000	<1,000
Nocardia asteroides 653	<10,000	<1,000
Sporotrichum schenkii	<10,000	<1,000
Trichophyton rubrum	<10,000	<1,000
Bacillus subtilis	<10,000	28,000,000
Staphylococcus aureus FDA 209	<10,000	21,000,000
Escherichia coli	<10,000	3,500,000
Pseudomonas aeruginosa ATCC 9027	<10,000	350,000

[*] Antibiotic diluted in agar medium (peptone 0.5%, glucose 1.0%, yeast extract 0.1%, agar 2.0%; pH 8.0) for the fungal spectrum and in liquid medium (peptone 0.75%, yeast extract 0.25%; pH 7.25) for the bacterial spectrum. Fungi incubated for 72 hours and the bacteria for 24 hours at 30 C.

[†] The cultures of fungal pathogens were obtained from Dr. N. F. Conant of Duke University.

[‡] Seven hundred and eight micrograms per milligram.

gested to us that *S. griseus* was producing, in addition to streptomycin, an antibiotic with antifungal activity.

The antifungal antibiotic was produced by Waksman's no. 4 strain of *S. griseus* in shaker flask cultures on the medium recommended by Waksman for the production of streptomycin (glucose, 1 per cent; meat extract, 0.5 per cent; peptone, 0.5 per cent; and NaCl 0.5 per cent). The preparation of this antibiotic that was used in determining its antibacterial and antifungal properties was obtained by extracting the beer of *S. griseus* with chloroform, removing the chloroform *in vacuo*, and dissolving the residue in methanol.

In contrast to streptomycin, the antifungal substance from *S. griseus* exhibits a high order of antibiotic activity against a number of yeasts and very little or no activity against the bacteria tested (table 1). It should be noted that of the fungal pathogens only *Cryptococcus neoformans* is highly sensitive to the action of this antibiotic. Furthermore, our antifungal antibiotic differs from streptomycin in its chemical properties. Streptomycin is chloroform- and ether-insoluble, whereas the antifungal antibiotic is chloroform- and ether-soluble; although both are water-soluble and thermostable.

At present, not enough information is available regarding the "second antibiotic of *S. griseus*" reported by Waksman (J. Bact., **51**, 753) and the antifungal agent from *S. griseus* to determine whether or not these two ether-soluble antibiotics are the same. Indicative of their being different is the relatively high degree of activity of Waksman's antibiotic against *Bacillus subtilis* (growth inhibited at 1:800,000 dilution). Strain differences between Waksman's and our *B. subtilis* test organisms, however, may explain this discrepancy in the anti-subtilis activity of the two antibiotic preparations.

A MODIFICATION OF HENRICI'S VEGETABLE-JUICE SPORULATION MEDIUM FOR YEASTS

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During a conference on alcohol production held at the Northern Regional Research Laboratory in February, 1943, the late Dr. A. T. Henrici discussed with us the infusion of four vegetables used by Mrak, Phaff, and Douglas (Science, **96**, 432) to obtain sporulation in yeasts. He informed us that he was using a commercially available blend of eight vegetable juices.[2] All that was required was the addition of sufficient agar to make a solid medium.

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[1] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

[2] The blend of vegetable juices used by Professor Henrici and by the writers was manufactured by Standard Brands at Terre Haute, Indiana, and marketed under the trade name, "V-8." We presume that other brands of mixed vegetable juice would prove equally satisfactory, and it is not our purpose to endorse any particular product.

We are using a modification of Henrici's medium made in the following manner. The contents of one can (designated as containing 1 pint, 2 fluid ounces) of the mixed juice from eight vegetables is adjusted to pH 6.8 with potassium hydroxide. One-half of a cake of compressed yeast is dispersed in it, and the mixture is steamed for 10 minutes to kill the yeast cells and liberate the acids which they possess. The medium is adjusted to pH 6.8 again and then added to an equal volume of hot distilled water containing 4 per cent of melted agar. The two solutions are mixed, then bottled or tubed, and sterilized for 15 minutes at 15 pounds' pressure. Unnecessary heating is avoided.

The medium is freshly slanted not more than 8 hours before use. The entire surface of the slant is lightly inoculated from a 24-hour culture grown on a yeast extract malt extract slant at approximately 28 C. The sporulation cultures are incubated at 24 to 25 C. Good sporulation of species of *Hansenula, Zygohansenula, Pichia,* and *Zygopichia* usually occur within 3 days, of *Saccharomyces* and *Zygosaccharomyces* at 5 to 7 days, and of *Debaryomyces* at 5 to 20 days.

The high degree of sporulation obtained, the year-round availability of the canned vegetable juice, and the ease with which the medium is prepared make it a very satisfactory sporulation medium for yeasts.

ANTISEPSIS AND IONIZATION OF SODIUM FLUORIDE

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Fifty years ago, Krönig and Paul (Z. Hyg. Infektionskrankh., **25,** 1) proved that strong mineral acids kill bacteria by their H ions, strong alkalies kill by OH ions, and heavy metal salts by their cations. It has been found since that the above-mentioned compounds were almost the only ones in which the ions caused death or bacteriostasis. With most disinfectants, organic or inorganic, the *undissociated* molecules are the active agents, whereas the ions are ineffective (Rahn and Conn: Ind. Eng. Chem., **36,** 185; Huntington and Rahn: J. Bact., **50,** 655). One notable exception has recently been found in the acridine dyes which act in proportion to their cations (Albert *et al.*: Brit. J. Exptl. Path., **26,** 160).

It seemed interesting to investigate whether hydrofluoric acid, the sodium salt of which is toxic, acts by its anion. A pure culture yeast was inoculated into glucose broth of different acidities containing various amounts of NaF.

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[1] This study was aided by the Necrochemistry Grant of The Wm. S. Merrell Company of Cincinnati.

pH	2	3	4	5	6
0.01% NaF	G	F	F	F	F
0.02% NaF	G	F	F	F	F
0.04% NaF	G	G	G	F	F
0.08% NaF	O	G	G	F	F
0.10% NaF	O	O	O	F	F

G indicates growth, but no fermentation, and F means growth and fermentation. Hydrofluoric acid proved to be most efficient in acid media in which it is undissociated. It behaved like H_2SO_3 and like the weak organic acids. As the dissociation constant of HF is 7.2×10^{-4} the greatest change in efficiency should occur between pH 2 and 5, and that is shown by the foregoing data.

At pH 7, a 2 per cent solution of NaF has no bactericidal power at all. With *Staphylococcus aureus*, the plate count showed

at start	6,400,000 cells
after 3 hours	6,100,000 cells
after 20 hours	3,100,000 cells

However, growth was retarded. The effect of dissociation of NaF was measured by adding NaCl, which decreased the concentration of F ions. The result with *S. aureus* in broth after 7 days was:

	0% NaCl	0.1% NaCl	0.4% NaCl	1.5% NaCl
0% NaF	+++	+++	+++	+++
0.2%	+++	+++	+++	+++
0.4%	++	+	0	0
0.8%	+++	0	0	0
1.5%	0	0	0	0

Again, the decrease of dissociation resulted in increased efficiency of the fluoride, proving that the undissociated fraction of the molecules was the cause of bacteriostasis.

SUMMARY

Sodium fluoride has no appreciable bactericidal power, not even in 2 per cent solution. Its bacteriostatic power depends upon the undissociated fraction of the molecules. In neutral solution the efficiency is increased by NaCl, in acid environment by increased H ion concentration.

AN ANTIFUNGAL SUBSTANCE FROM A STRAIN OF ASPERGILLUS FLAVUS[1]

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A strain (no. 70) of *Aspergillus flavus* was isolated which produced an antifungal substance in a 2 per cent tryptone, 0.5 per cent sodium chloride broth (table 1). No positive fungistatic action was observed until 72 hours after inoculation of the tryptone medium. From the third to the seventh day, there was a rapid rise in antifungal action of the filtrate, after which time it remained somewhat constant for about 2 weeks, and then decreased slightly in fungistatic power.

TABLE 1

Titer of a 14-day-old crude filtrate of Aspergillus flavus (no. 70) against 10 fungi

FUNGUS	TITER (IN DILUTION UNITS)*
Candida albicans (2091)	0
Nocardia asteroides (3308)	8
Microsporum gypseum (235)	8
Trichophyton mentagrophytes (598)	8
Trichophyton mentagrophytes (658)	16
Trichophyton mentagrophytes (640)	16
Trichophyton rubrum (661)	16
Trichophyton tonsurans (662)	16
Microsporum canis (9084)	16
Epidermophyton floccosum (1208)	32

* Incubation: 48 hours at 37 C.

A "dilution unit" is the inverse of the maximal dilution necessary to produce stasis.

Purified crystals from the filtrate, dissolved in 0.1 M sodium bicarbonate, were added in graded amounts to a 4 per cent glucose, 1 per cent Difco neopeptone broth, inoculated with standard spore suspensions of eight different dermatophytes and incubated 48 hours at 37 C. A concentration of 0.025 mg per ml of the crystals inhibited completely the growth of seven of the eight dermatophytes tested, namely, strains 9084, 598, 1208, 640, 662, 661, and 658; strain 235 alone required a concentration of 0.01 mg per ml before complete inhibition of its growth.

The sporocidal activity of the crystalline antibiotic was tested against strain 598. Normal saline solutions containing 12,000,000 spores per ml (from a 7-day growth at 37 C) received 5 mg and 1 mg of the antibiotic per ml, respectively, and were incubated at 37 C. After different exposure times, loopfuls were removed for viability tests to tubes each containing 5 ml glucose broth. Sporocidal activity was considered present if the exposed spores failed to develop within 10 days at 37 C. The higher concentration was 100 per cent sporocidal after 66 hours of contact, and the lower concentration after 98 hours. Phenol in a concentration of 5 mg per ml killed the spores in 26 hours, whereas a 0.1 M sodium bicarbonate control was innocuous.

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[1] This work was conducted while the author was on duty at the Naval Medical Research Institute, National Naval Medical Center, Bethesda, Md. The opinions or assertions contained herein are the private ones of the author and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

Comparative studies on the bacteriostatic, bactericidal, and fungicidal activities of the strain 70 crystals and of aspergillic acid indicated that the two antibiotics were similar, but probably not identical.

PATHOGENICITY OF CANDIDA SPECIES FOR THE CHICK EMBRYO

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Of the several medically important species of *Candida*, the pathogenicity of *Candida albicans*, as determined by injection into rabbits, has been well established by Benham (J. Infectious Diseases, **49**, 183). The animals die in 4 to 5 days, and characteristic lesions are found. In Benham's experiments other species of *Candida* produced no lesions in this animal. Stovall and Pessin (Am. J. Clin. Path., **3**, 347) found that *Candida tropicalis* would also produce lesions in rabbits if enormous doses were given. A new species, *Candida stellatoidea*, which produced no lesions in the rabbit, was introduced in 1937 by Martin, Jones, Yao, and Lee (J. Bact., **34**, 99). Some regard it as a dissociated form of *Candida albicans* (Martin and Jones: J. Bact., **39**, 609).

Moore (Am. J. Path., **17**, 103) has demonstrated that *Candida albicans* produced a marked infection of the chorioallantoic membrane of the developing chick embryo, subsequently causing death. We were interested in determining whether other medically important species of *Candida* exhibited a similar pathogenicity for the chick embryo.

Fertile Leghorn eggs were incubated for 10 days and were then processed according to the method described by Goodpasture and Buddingh (Am. J. Hyg., **21**, 319) as modified by Burnet (Med. Research Council, Special Rept. Series, no. 220, 1). A standard cell suspension was prepared by emulsifying the growth of a 2-day-old Sabouraud's agar culture and diluting to a definite turbidity, using a lumetron model 400-G photoelectric colorimeter (55 to 58 per cent transmission). One capillary drop of the suspension was used to inoculate each embryo.

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The infected embryos were incubated at 35 C and were observed daily until the eighth day when all surviving embryos were sacrificed and examined. All embryos dying earlier were autopsied on the day of death. All but one of the uninoculated controls survived the observation period.

Three laboratory strains of *Candida albicans* were used to inoculate the chorioallantoic membrane of 17 chick embryos. The lesions were apparent at the end of 24 hours, reached maximum intensity at 48 to 72 hours, and remained so until death of the embryo. These lesions were very marked and consisted of either one large, yellowish ulcer 10 to 20 mm in diameter or several large, discrete, thick, yellowish-white raised areas. The membrane was thickened and puckered. Sixteen died in from 2 to 5 days, the majority on the fourth or fifth day. One lived until the eighth day. Many of the embryos were hemorrhagic when autopsied.

All five chick embryos infected with *Candida stellatoidea* died on the fifth day. Grossly, lesions were very similar to those produced by *Candida albicans.*

Three strains of *Candida tropicalis*, 2 stock and 1 freshly isolated, were used to inoculate 12 embryos. Five survived until sacrificed, whereas seven died between the second and sixth days. The lesions were mild in character and ranged from a few discrete, yellowish-white raised areas to small ulcers with the membrane slightly puckered.

Five embryos were inoculated with *Candida krusei* and four each with 2 strains of *Candida parakrusei.* Neither of these two species produced true lesions, but some membranes were slightly puckered or showed a slight opacity, which disappeared in 48 to 72 hours. After this time the membranes appeared normal, and little or no evidence of inflammation was present when all of the embryos were sacrificed on the eighth day.

Each of the infected embryos was cultured and autopsied at death or when sacrificed. Cultures prepared from various parts of the embryo suggested that *Candida albicans* was able to invade other parts of the embryo following an infection of the chorioallantoic membrane. The results indicate that *Candida albicans* and *Candida stellatoidea* caused severe lesions to develop on the chorioallantoic membrane and the infection proved fatal to the embryo. Lesions produced by *Candida tropicalis* were mild in character but the infection apparently caused the death of about one-half of the embryos. The other strains used, *Candida krusei* and *Candida parakrusei*, neither caused lesions nor death of the embryos.

THE APPLICATION OF WALKER'S INDEX OF FUNCTIONAL NORMALITY TO A STUDY OF DEVELOPING CHICK EMBRYOS INFECTED WITH THE LEVADITI STRAIN OF VACCINIA

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Two methods are generally employed for analyzing results obtained in studies of the growth of viruses on developing chick embryos: (1) counting of pocks and changes in the structure of the egg membranes and (2) estimation of the percentages of deaths among the embryos. No attempts have previously been made to record the changes of functional normality of the developing chick during the experiments. In most cases the morphology of embryos observed when the eggs were candled has been used to determine the age and development of the embryos. This procedure has not always been reliable because the source of the eggs is often a commercial hatchery and the eggs are not from controlled flocks.

Walker (1938), in a study of normal stages of the development of chick embryos, suggested the use of the crown-rump length in conjunction with the wet weight, expressed as index values, as a criterion for determining variation in functional normality. He used the formula $I = \frac{1{,}000\sqrt[3]{W}}{L}$ to calculate these values. In the study of 361 normal Barred Plymouth Rock chick embryos of 7 to 19 days' incubation age, he found a reasonably good frequency curve could be set up, with a mean represented by index values in frequency classes 333 to 335 and a range with extreme limits in frequency classes of 315 to 317 and 351 to 353. Any embryos outside this range he considered abnormal. In our studies of developing chick embryos inoculated by the allantoic route with the Levaditi strain of vaccinia, the Walker formula was applied as a means of analyzing some of the data obtained.

EXPERIMENTAL METHODS

All of the eggs in these experiments were from New Hampshire Red chickens and were obtained from a commercial hatchery where they had been incubated in a Buckeye "forced draft" type incubator. Eggs were candled at the hatchery and were packed at incubator temperature in containers heavily lined with cotton. They were then transferred to the laboratory incubator, the temperature of which was 39.5 C. On the day prior to use, the eggs were opened by the Goodpasture and Buddingh technique (1935) and returned to the incubator.

In these experiments two lots of eggs were used. They will be designated as series A and B. Series A contained 223 normal embryos and 90 infected embryonated eggs. The range in age for normal and infected eggs was the same, 10 to 18 days. The normal embryos were used in part as incubator controls for the infected eggs and the rest as a source of allantoic and amniotic fluid in another phase of the experiment. Ninety embryonated eggs were inoculated by the allantoic sac method, with 0.1 ml of a 1:150,000 dilution of the Levaditi strain of vaccinia, in order to ensure six living embryos for each of the 9 days of the experiment. Fifty-four infected eggs were studied.

Series B contained 174 normal eggs and 179 infected eggs. The latter were used to subculture the virus in an experiment to determine the effect *in vitro* of allantoic fluid and amniotic fluids on the virus. They were of the same age at the time of inoculation, 10 days, and were 12 days old when sacrificed. The chorio-allantoic membrane route of Goodpasture and Buddingh (1935) was used for the inoculations in series B.

TABLE 1

Tabulation of average values reported for normal chick embryos

DAY	WEIGHT			LENGTH		pH ALL. FL.		pH AMN. FL.		LN. WT		
	(1)	(2)	(3)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(3)
10	2.4	2.0	2.6	3.8	3.7	7.68	7.49	7.83	6.90	0.81	0.64	0.93
11	3.4	2.8	3.4	4.3	4.2	8.71	7.31	7.75	6.83	1.22	1.04	1.34
12	5.2	3.8	5.2	5.0	4.7	7.96	7.39	7.72	6.65	1.63	1.34	1.64
13	7.7	5.2	7.3	5.8	5.2	7.98	7.39	7.52	6.58	2.03	1.64	1.99
14	9.8	7.4	10.4	6.2	5.8	8.00	6.79	7.54	6.54	2.27	2.01	2.34
15	13.3	10.0	13.8	6.9	6.4	7.37	5.87	6.90	6.40	2.58	2.31	2.62
16	15.3	12.8	15.7	6.9	7.1	6.80	5.75	6.80	6.92	2.72	2.54	2.76
17	17.5	14.9	18.8	7.5	7.4	6.28	5.60	6.89	7.12	2.85	2.70	2.94
18	22.5	17.3	22.3	8.2	7.8	5.85	5.71	7.17	7.48	3.11	2.85	3.10

Investigators are indicated by number at the heads of the columns as follows: (1) Hoffstadt and Tripi; (2) P. A. Walker; (3) Romanoff and Romanoff.

Legend: All. Fl. indicates allantoic fluid; Amn. Fl. indicates amniotic fluid; LN. Wt indicates the natural logarithm of the weight.

When the eggs were to be sacrificed, the allantoic and amniotic fluids were removed with a 2-ml syringe fitted with a 27-gauge ½-inch needle, and extreme care was taken not to puncture blood vessels. The embryos were then placed in sterile petri dishes and freed of their extraembryonic membranes, wet weights were taken, and crown-rump length measurements were made. The pH's of the allantoic and amniotic fluid were determined for all embryos by the use of the Beckman pH meter.

EXPERIMENTAL RESULTS

Rate of growth. In comparing the results obtained in this study of normal New Hampshire Red embryos with those of Walker (1943*a*, 1943*b*, 1943*c*) for normal embryos of Barred Plymouth Rocks, it was found that the crown-rump lengths correlated more closely than did the weights. The average daily weight of the embryos was more nearly that reported by Romanoff and Romanoff (1933) for White Leghorns (table 1).

Despite the discrepancies noted for daily gain in weight, the over-all percentage gain showed a fairly close correlation: Walker, 31 per cent; Romanoff and Romanoff, 32.5 per cent; and that obtained by us, 32 per cent. This may be better demonstrated by the representation of the cumulative weights of embryos expressed as the natural logarithms of the average weights (figure 1). It is readily apparent that all three curves are similar in shape and slope.

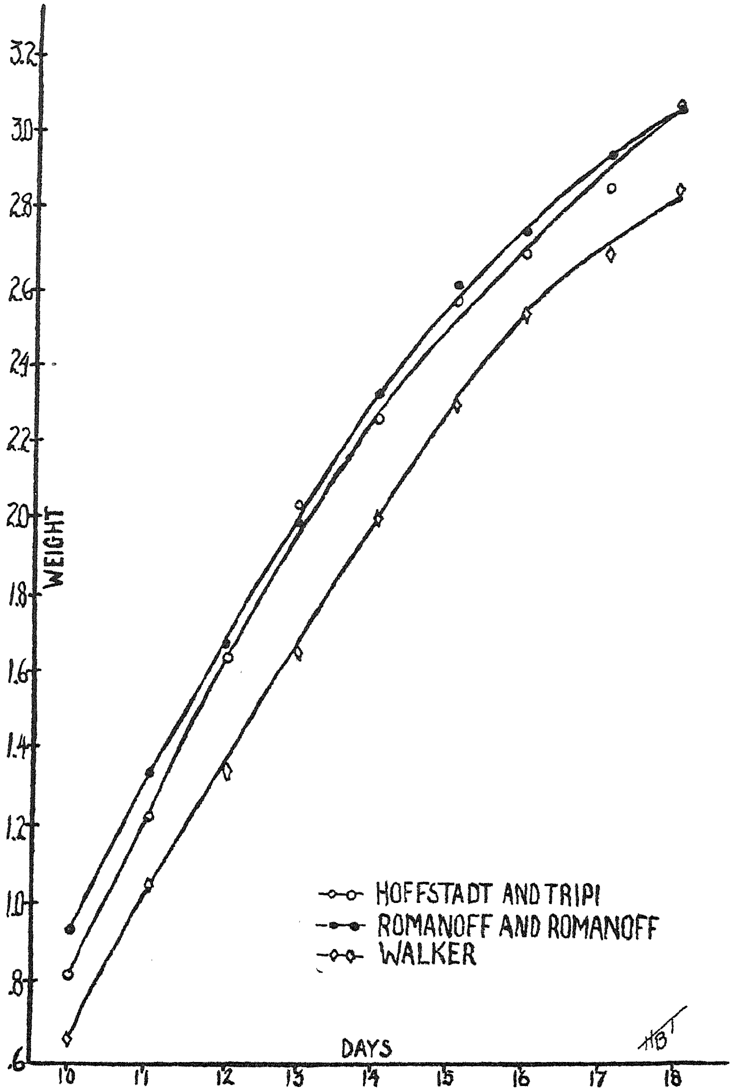

Fig. 1. Cumulative Growth Curves for Normal Embryos
Weight expressed as its natural logarithm

Functional normality. The index values for functional normality for 223 normal embryos ranging from the age of 10 to 18 days were calculated by the formula as suggested by Walker: $I = \frac{1{,}000 \sqrt[3]{\text{wet weight in grams}}}{\text{crown-rump length in cms}}$. The results of application of the index are illustrated in the histogram (figure 2). Embryos used in this experiment differed in breed from those used by Walker, were from a smaller age group, and probably came from a number of different flocks of chickens. As is to be expected under these conditions, greater variations in the indexes were encountered, yet the absolute range, 290 to 414, differed but 2 units from the 294 to 416 of Walker. Because fewer embryos (223) were available for this determination, and because there was greater variation toward the two extremes, an interval of 5 units, rather than 3 units as used by Walker, was selected to show index frequency. Absolute limits were 290 and 414.

From the histogram, it is apparent that a reasonably good frequency curve could be constructed. Since the indexes tend to be concentrated toward the higher values, the curve would be positively skewed and would be characterized by the following values:

Arithmetic mean	347.72
Median	347.02
Mode	346.22
Standard deviation	17.76
Coefficient of variation	5.11
Coefficient of skewness	0.0845
Standard error of the mean	1.19
Range of normality (including approximately 95.5 per cent of all embryos)	310–384

The range of normality was determined by using 2 standard deviations rather than the 3 standard deviations usually used, because of the wide frequency distribution, which was assumed to be due to the lack of control of the source of the eggs.

Two factors may account in part for this variation: (1) the age of the embryos (7- to 19-day-old embryos were used in Walker's experiment and 10- to 18-day-old embryos were used by us); (2) the breed of the embryos (embryos of New Hampshire Reds were uniformly heavier than the embryos of Barred Plymouth Rocks used by Walker).

Rate of growth of the embryos following inoculation of vaccinia (Levaditi strain) into the chorioallantoic fluid. Of the 90 eggs inoculated with the virus, 6 living embryos were sacrificed daily from the 10th to 18th day of incubation of the embryos. A total of 54 embryonated eggs was studied (table 3). The embryos were weighed and measured, and the allantoic fluids and organ extracts were each subcultured on three eggs. Both the crown-rump length and the wet weight showed fluctuations from the normal. The over-all percentage gain in daily weight was 26.9 per cent as compared with 32 per cent for the normal embryos. The actual difference in the final average weight was 6.5 grams less for the infected embryos than the normal ones. Cumulative growth curves for normal and in-

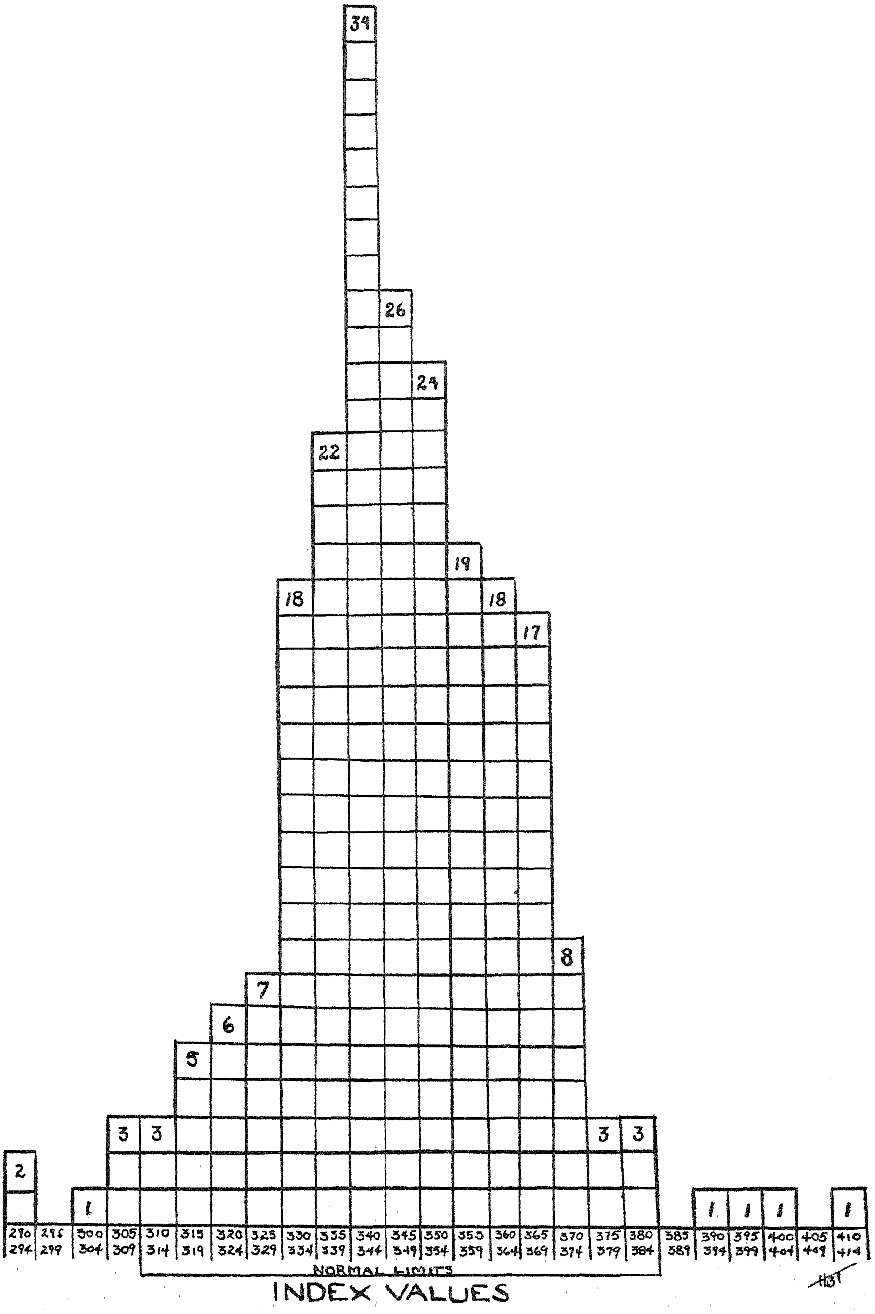

FIG. 2. HISTOGRAM RESULTING FROM APPLICATION OF WALKER'S INDEX OF FUNCTIONAL NORMALITY TO NORMAL EXPERIMENTAL EMBRYOS, SERIES A

fected embryos are shown in figure 3. The smallest gains in weight by the embryos were on the 12th, 15th, and 18th days.

Walker (1943) reported a definite shift to the acid side in the pH of the allantoic fluid in normal embryos, and this was verified by our results, although the drop was not so sharp as that reported by Walker (table 1). This increase in acidity foreshadows the conditions seen in adult avian urine (Takamatsu, 1935). A similar change in the pH of the amniotic fluid was observed on the 14th and 15th days (table 3). The average difference in length between the normal and infected embryos was 1.8 cm (tables 2 and 3).

TABLE 2

Average normal values for chick embryos—series A*

DAY	WEIGHT	LENGTH	ALL. FL. pH	AMN. FL. pH	INDEX	LN. WT	% GAIN OF WT
	gm	*cm*					
10	2.4	3.8	7.68	7.83	349	0.81	—
11	3.4	4.3	7.71	7.75	348	1.22	41.6
12	5.2	5.0	7.96	7.72	348	1.63	53.0
13	7.7	5.8	7.98	7.52	342	2.03	48.1
14	9.8	6.2	8.00	7.54	343	2.27	27.3
15	13.3	6.9	7.37	6.90	343	2.58	35.7
16	15.3	6.9	6.80	6.80	356	2.72	15.0
17	17.5	7.5	6.28	6.89	348	2.85	14.3
18	22.5	8.2	5.85	7.17	346	3.11	28.6

Legend: Same as for table 1.
* Averages of 25 embryos per day.

TABLE 3

Average values for infected chick embryos—series A*

DAY	WEIGHT	LENGTH	ALL. FL. pH	AMN. FL. pH	INDEX	LN. WT	% GAIN OF WT
	gm	*cm*					
10	2.2	3.5	7.99	7.84	370	0.80	—
11	3.3	4.1	8.33	7.31	360	1.18	48.0
12	3.8	4.3	8.29	7.59	364	1.34	17.1
13	5.0	4.8	7.96	7.08	353	1.61	31.3
14	7.8	5.4	7.76	7.24	368	2.05	56.1
15	9.0	6.1	8.02	7.16	342	2.19	14.0
16	11.9	6.5	7.10	6.67	353	2.47	32.7
17	14.8	7.2	6.17	6.68	341	2.68	24.4
18	16.0	7.4	6.53	7.41	341	2.76	7.5

Legend: Same as for table 2.
* Average of 5 embryos per day.

Functional normality. Walker's index for functional normality was calculated for the infected embryos. Figure 4 is the histogram obtained for the infected embryos of series A. Three embryos were outside the normal range for functional normality previously established. Calculations made upon these frequencies characterize a curve with the following values:

Arithmetic mean	353.33
Median	352.92
Mode	352.10
Standard deviation	15.38
Coefficient of variation	4.35
Coefficient of skewness	0.080
Standard error of the mean	2.11
Range of normality, including approximately 95.5 per cent of all embryos	325–384

Comparison of the foregoing values with those obtained for normal embryos shows the following relationship:

Standard error of differences between means (σ_D)	2.42
Critical ratio (T)	$2.73\sigma_D$

The critical ratio, $2.73\sigma_D$, is above the 1 per cent level of significance ($2.576\sigma_D$) and it may be concluded, therefore, that there is a significant difference between the two means which cannot be attributed to random errors of sampling. Actually, the probability that differences so large could occur as the result of random causes is less than 1 in 100. From application of the index of functional normality to the experimental material and comparison with normal values it is concluded that the functional normality of the infected embryos was altered. The most significant factor was the shift of the mean from the 340-to-344 to the 350-to-354 frequency class.

Series B embryos. Since it was apparent that the Walker index could be applied for the interpretation of data over a period of several days of embryonic growth, it was applied to embryos of a single age group. The infected eggs, 179 in number, were inoculated by the chorioallantoic membrane route on the 10th day and were sacrificed on the 12th day. The normal controls were 174 in number. The results of the application of Walker's index of functional normality to the experimental material are shown by the histograms (figures 5 and 6) and by calculated values for the frequency curves as follows:

	Normal	*Infected*
Arithmetic mean	347.7	353.4
Median	347.3	352.4
Mode	346.5	350.4
Standard deviation	19.51	19.98
Coefficient of variation	5.61	3.66
Coefficient of skewness	0.112	0.150
Deviation of the mean	1.88	1.50
Range of normality including approximately 95.5 per cent of all embryos	308.7–386.7	313.4–393.4
Standard error of differences between means (σ_D)	2.107	
Critical ratio	$2.70\sigma_D$	

With the critical ratio of $2.70\sigma_D$, inspection of the probability tables reveals the fact that the probability that the differences between means could be so great as a result of random errors of sampling would be less than 1 in 100. The difference may, therefore, be said to be a significant one. It is of significant interest that these values closely approximate those calculated for the experimental material of series A embryos.

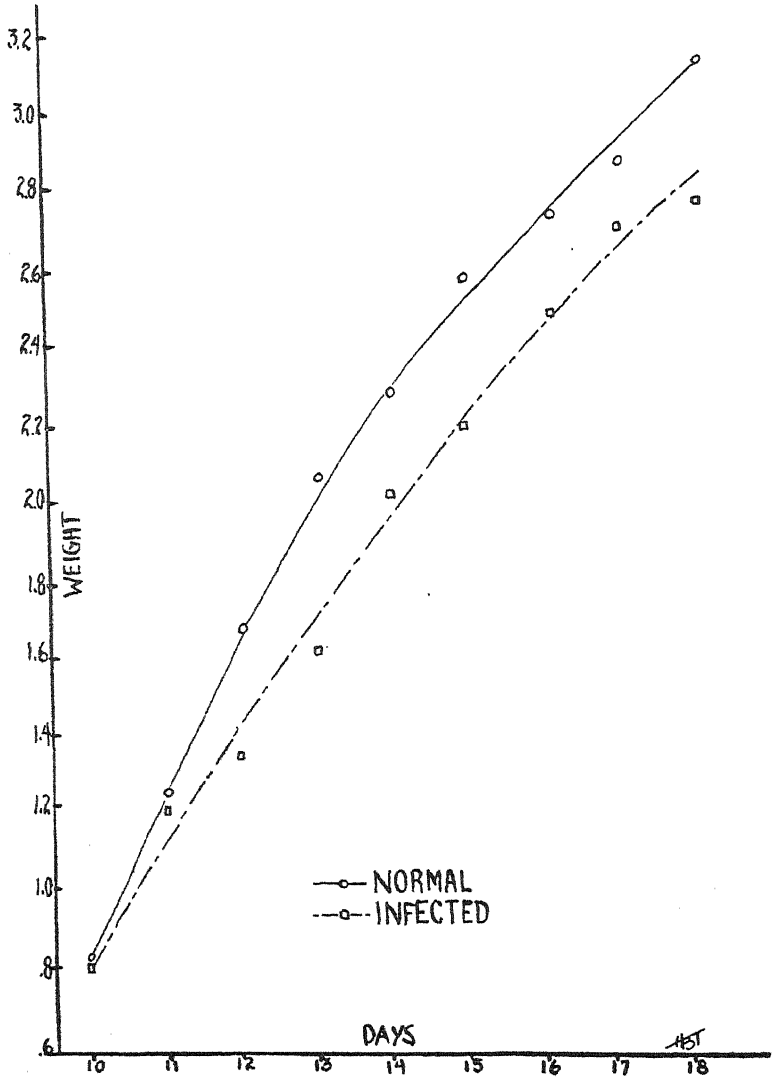

FIG. 3. CUMULATIVE GROWTH CURVES FOR NORMAL AND INFECTED EMBRYOS
Weight expressed as its natural logarithm

SUMMARY

The over-all daily rate of growth, expressed as percentage gain in weight of allantoic-sac-infected embryos, was 26.5 per cent, as compared with the normal

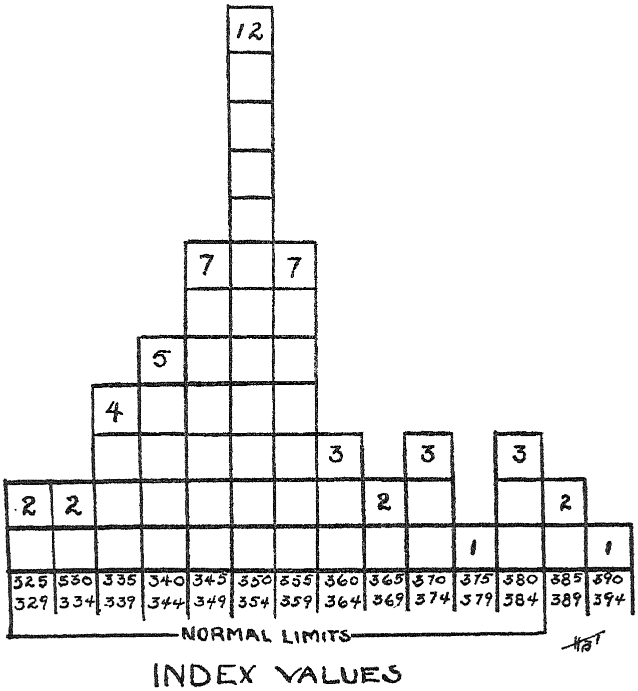

FIG. 4. HISTOGRAM RESULTING FROM APPLICATION OF INDEX TO INFECTED EXPERIMENTAL EMBRYOS, SERIES A

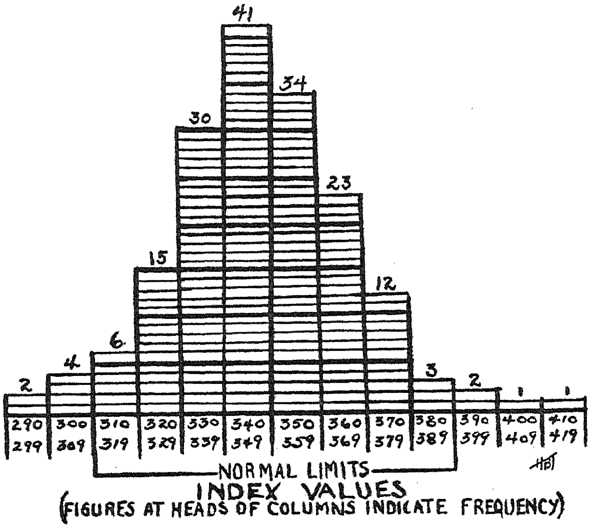

FIG. 5. HISTOGRAM RESULTING FROM APPLICATION OF INDEX OF FUNCTIONAL NORMALITY TO NORMAL EXPERIMENTAL EMBRYOS SERIES B

rate of 32 per cent. A normal rate of growth was maintained for allantoic-sac-infected embryos for the 10- to 11-day period but was followed by a loss of rate of growth in the 12th, 15th, and 18th days. The final average weight of infected embryos on the 18th day did not equal that of normal 17-day-old embryos. The actual final discrepancy between the infected and normal embryos was 6.5 grams.

Comparison of the functional normalities of infected embryos and normal embryos, as expressed by the Walker index, indicates an alteration of functional normality for the infected embryos.

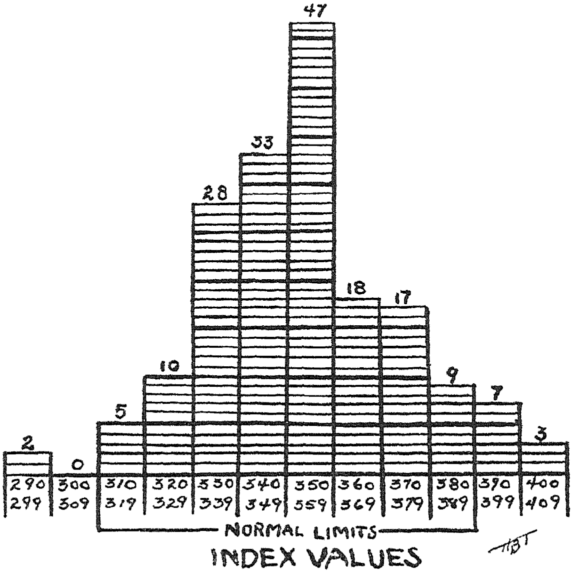

FIG. 6. HISTOGRAM RESULTING FROM APPLICATION OF INDEX OF FUNCTIONAL NORMALITY TO EXPERIMENTALLY INFECTED EMBRYOS, SERIES B

Comparison of the functional normalities of embryos infected by either the allantoic fluid or the chorioallantoic membrane route indicates no difference in the degree of alteration of functional normality obtained by either method of inoculation.

Comparison of the results of application of the index of functional normality to 10- to 18-day embryos and to 12-day embryos indicates that the Walker index is applicable to embryos of a single age or of a range of ages.

The numbers of eggs used for these determinations varied and may, in part, account for some discrepancies in calculations, but the discrepancies were all within the limits of error.

From these results it may be concluded that the Walker index is of value for interpreting the functional normality of infected embryos.

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TWO NEW AMINO-ACID-FERMENTING BACTERIA, CLOSTRIDIUM PROPIONICUM AND DIPLOCOCCUS GLYCINOPHILUS

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Previous papers from this laboratory have described the isolation from garden soil or marine mud of several types of anaerobic bacteria capable of fermenting certain amino acids and other nitrogenous compounds (Barker, 1937, 1939, 1943; Barker and Beck, 1942). The present paper deals with the isolation and characterization of two anaerobes that were obtained from black mud by the enrichment culture method using media containing alanine and glycine, respectively, as fermentable substrates (Cardon, 1942).

THE ALANINE-FERMENTING BACTERIUM

The isolation of the alanine-fermenting bacterium was accomplished by the use of a medium containing alanine, 1; yeast autolysate, 1 vol.; M/1 phosphate buffer pH 7.0, 2 vols.; and tap water, 100. The medium was heavily inoculated with black mud from San Francisco Bay and incubated at 37 C in a completely filled glass-stoppered bottle. Within 48 hours the medium became turbid. The dominant organism was a motile, gram-negative rod. After 5 days all the amino nitrogen had been converted to ammonia. There was no change in pH, since volatile acids were formed in amounts equivalent to the ammonia. Distillation of the volatile acids by the Duclaux method indicated a 2:1 mixture of propionic and acetic acids.

After two transfers in the same liquid medium, the organism was isolated by the shake culture method. For this purpose the yeast autolysate was increased to 3 volumes per cent to provide more adequate nutrition without unduly favoring the common putrefactive anaerobes. No special difficulty was encountered in the isolation.

In young cultures the alanine-fermenting organism appears as a motile, gram-negative, characteristically spindle-shaped rod, occurring singly or more commonly in pairs (figure 1). The average size is 0.8 by 3.0 microns. In older cultures the cells vary considerably in size and shape. Spores do not form readily. Even in old stab cultures, in which spores form most abundantly, seldom do more than a third of the cells sporulate. The spores are oval and about one-third as long as the sporangium (figure 2). They are terminal or subterminal, cause a slight swelling of the cell, and separate from the sporangium soon after being formed. The motility is due to 3 to 5 peritrichous flagella.[2]

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[2] The authors are indebted to Dr. J. V. Bhat for making the flagellar stains.

The organism is an obligate anaerobe and is catalase-negative. It grows abundantly in 16 to 24 hours at 28 to 37 C in a favorable medium. Liquid media first become uniformly turbid and then gradually clear after 3 to 4 days. Deep agar colonies are lens-shaped with smooth edges.

Rapid and abundant growth of pure cultures can be obtained in the following medium: alanine, 0.3; bacto peptone, 0.3; bacto yeast extract, 0.4; M/1 phosphate buffer pH 7.1, 0.5 vol.; saturated solution of calcium sulfate, 0.25 vol.; cysteine hydrochloride, 0.02; $MgSO_4 \cdot 7H_2O$, 0.005; $FeSO_4 \cdot 7H_2O$, 0.001; and distilled water, 100. Growth will occur over the pH range 5.8 to 8.6, the optimum being between 7.0 and 7.4. Cysteine may be replaced as a reducing agent by sodium sulfide. Thioglycolate should not be used since it is somewhat inhibitory, particularly when the concentration is above 0.02 per cent. A complex nutrient such as yeast extract is essential; almost no growth occurs in its absence. The addition of peptone is beneficial but not essential. Even with yeast and peptone, growth is very poor in the absence of alanine or one of the related compounds such as lactate, pyruvate, acrylate, serine, or threonine, which are readily fermented. Glucose is not attacked by this organism.

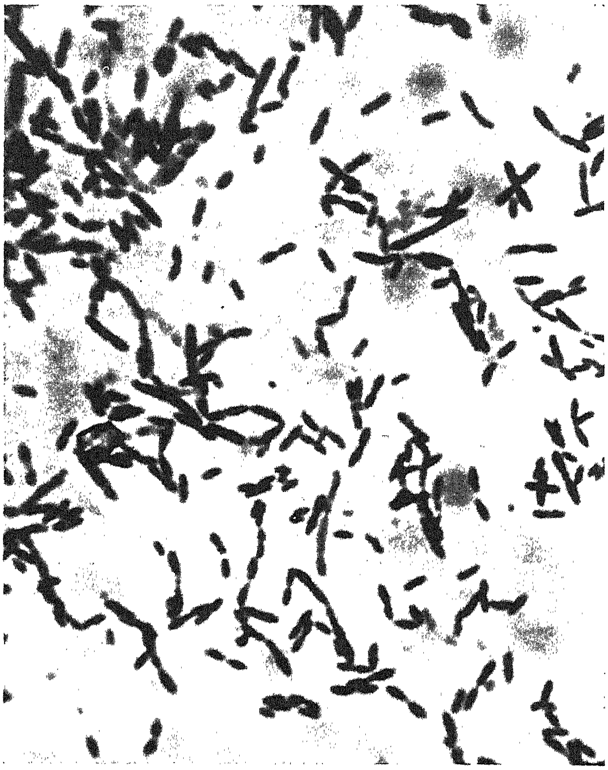

FIG. 1. CLOSTRIDIUM PROPIONICUM

Strain X2. From 3-day-old culture in liquid alanine, peptone, yeast extract medium. Erythrosin stain. X 1,000.

The fermentation of alanine and related compounds is of the propionic acid type. The equation for the decomposition of alanine is

$$3\ CH_3CHNH_2COOH + 2\ H_2O = 3\ NH_3 + 2\ CH_3CH_2COOH + CH_3COOH + CO_2.$$

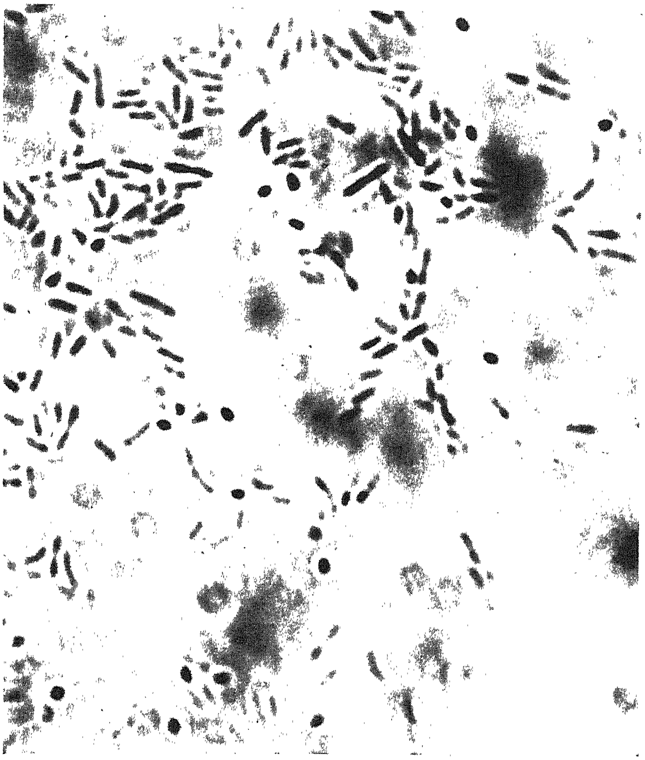

FIG. 2. CLOSTRIDIUM PROPIONICUM, SHOWING SPORES

Strain X2. From 7-day-old colony on an anaerobic slant of alanine, peptone, yeast extract agar. Unstained. Slightly out of focus. X 1,800.

No hydrogen or succinic acid is formed. Further data on the chemical activities of this organism will be presented in another place.

Since no *Clostridium* with similar characteristics appears to have been described previously (Bergey *et al.*, 1939), we have decided to create a new species for the alanine-fermenting organism. The name *Clostridium propionicum* was chosen as being indicative of its catabolism.

THE GLYCINE-FERMENTING ORGANISM

The enrichment and isolation of the glycine-fermenting organism was accomplished by the same general methods used for *Clostridium propionicum*. The first enrichment culture medium, consisting of 1 per cent glycine in tap water heavily inoculated with bay mud, gave a vigorous fermentation in 3 days at 37 C. All the amino nitrogen was converted to ammonia, and carbon dioxide and acetic acid were formed. There was also a considerable production of methane in the first and second enrichment cultures. In subsequent transfers methane production decreased, and none at all was formed by pure cultures of the glycine-fermenting organism. For transferring the enrichment culture, the original medium was supplemented with the usual salts and 1 volume per cent of yeast autolysate.

The predominant organism in all the enrichment cultures was a rather large coccus. After three transfers the coccus was isolated by using a solid medium containing 0.5 per cent glycine, 3 volumes per cent yeast autolysate, and salts. Five strains were obtained which were all very similar in morphology and physiology.

The coccus is strongly gram-positive.[3] The cells vary considerably in size even in young cultures, the range being from 0.7 to 2.5 microns in diameter, the average being about 1.2 microns. In every culture a few of the cells are definintely rod-shaped. The cells usually occur in pairs and in short, irregular 4- to 6-cell chains (figure 3). The irregularity of the chains suggests that the organism may be capable of dividing along two or more axes. Small clusters of cells also occur.

The organism is an obligate anaerobe which does not grow to an observable extent on complex nitrogenous or sugar-containing media in the absence of added glycine. Slow but abundant growth occurs in a medium containing glycine, 0.3; bacto yeast extract, 0.5; bacto peptone, 0.5; and the other constituents used in the medium for *Clostridium propionicum.* Since ordinary distilled water is frequently toxic to this organism, it is best to use glass-distilled water or a 1:1 mixture of glass-distilled and tap water. The optimum pH for growth in this medium is about 7.2, the range being from 6.0 to 8.5. An incubation of 48 to 72 hours is required to obtain moderately heavy growth at 30 C. Growth is a little faster at 37 C.

In liquid media, the glycine-fermenting coccus grows mainly as a sediment on the bottom or along the lower walls of the container, leaving the solution clear. Deep agar colonies are white to cream-colored, and spherical with a rough surface.

The cells show a moderate catalase activity.

The organism obtains energy by a fermentation of glycine according to the equation

$$4\ CH_2NH_2COOH + 2\ H_2O = 4\ NH_3 + 3\ CH_3COOH + 2\ CO_2.$$

In a fermentation balance experiment the carbon recovery was 97 per cent and the redox index 0.99. In stationary cultures no appreciable amount of hydrogen is produced, and no other gas is evolved since the carbon dioxide is retained as bicarbonate. Under other conditions, however, when a culture is shaken in contact with a large nitrogen-filled gas space, as much as 50 mM of hydrogen are formed per 100 mM of glycine fermented, and there is a corresponding increase in the yield of carbon dioxide and decrease in acetic acid.

Some 35 compounds were tested to see whether they can replace glycine as a fermentable substrate. These included all the common amino acids, and nonnitrogenous substrates such as glucose, pyruvate, and glycolic acid. No substitute was found for glycine; serine and pyruvate are attacked very slowly but only in the presence of glycine, not in its absence. A more complete discussion of the biochemical activities of the organism will be given elsewhere.

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[3] A previous statement (Cardon, 1942) that the organism is gram-negative is erroneous.

The classification of the glycine-fermenting coccus is not a simple matter. The organism might be placed in any one of our different genera (*Micrococcus*, *Staphylococcus*, *Diplococcus*, or *Streptococcus*) in either of two families (*Micrococcaceae* or *Lactobacteriaceae*) depending on the degree of emphasis given to various details of its morphology.[4] However, since pairs of cells and short

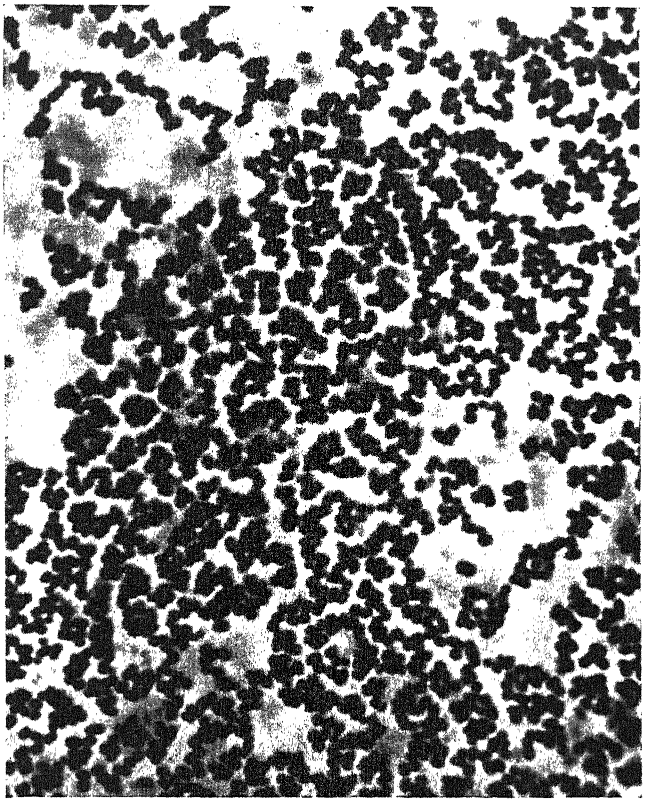

FIG. 3. DIPLOCOCCUS GLYCINOPHILUS

Strain R1. From 3-day-old culture in liquid glycine, peptone, yeast extract medium. Erythrosin stain. X 1,000.

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[4] In view of the uncertainty involved in classifying gram-positive, anaerobic cocci on the basis of the finer and more elusive points of morphology, it would seem desirable to place all of these organisms that exhibit a more or less irregular and variable arrangement of the cells and a chemoheterotrophic type of catabolism in a single genus that might be called *Anaerococcus*. This would perhaps be only a temporary expedient, but it would serve a useful purpose in the present state of knowledge. Since this is not a suitable place to develop a new classification of this group, we wish only to leave this suggestion for the consideration of future taxonomists.

chains are particularly characteristic of this organism, we have decided to place it in the genus *Diplococcus* in spite of the fact that it differs from other members of the genus in producing the enzyme catalase.

No organism identical with the glycine-fermenting coccus appears to have been previously described (Bergey *et al.*, 1939; Prévot, 1933; Hucker, 1924). Bergey lists two anaerobic species of *Micrococcus, M. grigoroffi* and *M. niger*, both of which differ markedly from our organism in morphology and nutrition. The same applies to the three anaerobic members of the genus *Staphylococcus* listed in Bergey's manual (*S. aerogenes, S. anaerobius,* and *S. assaccharolyticus*). *Gaffkya anaerobia* and the three anaerobic species of *Sarcina* (*S. beijerinckii, S. maxima,* and *S. methanica*) are easily differentiated from the glycine-fermenting coccus on the basis of morphology alone. In the genus *Streptococcus*, seven anaerobic species are listed by Bergey. Six of these (*S. intermedius, S. micros, S. lanceolatus, S. putridus, S. foetidus,* and *S. anaerobius*) can be eliminated immediately because, unlike the glycine-fermenting coccus, they produce acid from glucose and other sugars. The seventh species, *S. parvulus*, differs in respect to its small size and black colonies. Of the five anaerobic species in the genus *Diplococcus* only *D. magnus* shows some similarity to our organism. However, the two are certainly not identical since *D. magnus* is appreciably larger (1.5 to 1.8 microns) and grows readily in broth and peptone water.

Since it is evident that the glycine-fermenting coccus represents a new species, we propose the name *Diplococcus glycinophilus*.

SUMMARY

Two new obligately anaerobic bacteria capable of fermenting single amino acids have been isolated from marine mud by the enrichment culture method. *Clostridium propionicum* causes a propionic acid type fermentation of alanine, serine, lactate, and other related compounds. *Diplococcus glycinophilus* ferments glycine and is apparently unable to use any other compound as an energy source.

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A BACTERICIDAL DETERGENT FOR EATING UTENSILS[1]

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A good detergent should depress the interfacial tension at a water-oil interface below 5 dynes per cm (Snell, 1932), and in addition it should have good deflocculating and dispersing power (Snell, 1933). As detergents, anion-active agents are generally superior to cation-active and nonionic agents, but as bactericides, cationics are superior. An ideal dishwashing compound would be one that combines the detergent properties of the anion-active agents with the bactericidal properties of the cation-active. Since these two types of surface-active agents are incompatible, it is impossible to combine them, because they would neutralize each other with the formation of an insoluble precipitate, resulting in the loss of detergent as well as bactericidal properties. The purpose of this investigation was to develop a detergent suitable for washing and sanitizing eating utensils.

EXPERIMENTAL

Two detergent compositions were prepared, one with and one without a cation-active bactericide.

	No. 1	*No. 2*
Trisodium phosphate	33	33
Sodium carbonate	33	33.5
Borax	33	33.5
Ethyl cetab[2]	3	
Nonionic wetting agent[3]	1.5	3.0
	103.5	103.0

An artificial soil was prepared according to Gilcreas and O'Brien (1941), consisting of:

100 g	raw eggs (white and yolks mixed)
20 g	butter
20 g	lard
20 g	peanut butter
20 g	milk
10 ml	*Staphylococcus aureus* culture (24-hour)
190 g	

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[1] This investigation was supported by the Rhodes Chemical Corporation, Philadelphia, Pennsylvania.

[2] Cetyl dimethyl ethyl ammonium bromide (Rhodes Chemical Corporation).

[3] An alkylated aryl polyether alcohol.

Twenty-four clean microscope slides were immersed in the artificial soil at 37 C. They were then removed with forceps, and 12 slides each were placed in two monel metal staining racks (Fisher Scientific Company, catalogue no. 8-820) and allowed to drain and dry in the air at room temperature for one-half hour.

Two detergent solutions were prepared with formula no. 1 and formula no. 2 by dissolving 3 g of each respectively in 400 ml distilled water (1 oz per gallon). The solutions were warmed to 37 C and maintained at that temperature throughout the test. The pH of both solutions was 11.5.

After the contaminated slides had dried for one-half hour, one rack was placed in detergent no. 1 and the other in detergent no. 2. The slides were completely covered by the detergent solutions. Each rack was then repeatedly raised out of and then reimmersed in the solution about once a second for a period of 10 minutes. At intervals of 1, 2, 3, 4, 5, and 10 minutes two slides were removed from each rack and placed in separate petri dishes containing 20 ml of sterile FDA broth. At the end of the 10-minute test period all petri dishes were gently agitated to insure mixing, and 1-ml portions were withdrawn from each under sterile conditions. Pour plates were then made with these portions in FDA nutrient agar, using 1 ml each of three dilutions, 1:10, 1:100, and 1:1,000. All petri dishes containing the original microscope slides as well as all pour plates were incubated at 37 C for 24 hours. They were then examined for bacterial growth, and the number of colonies on each pour plate was counted.

It was found that formula no. 2 (without ethyl cetab) is not bactericidal, although there was a gradual drop in the bacterial count, probably due to the alkalinity of the solution. Formula no. 1 produced a very marked sharp drop in the bacterial count up to the 5-minute washing period, at which point the pour plates and the broth were sterile; but at the 10-minute washing period there was a heavy growth of *Staphylococcus aureus* in the broth as well as on the agar plates.

We believe that the explanation for this apparent anomaly is as follows: The bacteria were thoroughly distributed throughout the artificial soil, which consisted of over 30 per cent fat. The slides were then immersed in this soil, which was then allowed to dry on the slides. When the slides were placed in the detergent solution, the detergent quickly removed the soil from the slides and emulsified the fat. The ethyl cetab killed the bacteria in the aqueous phase of the detergent solution and on the clean slides, but did not kill the bacteria which were entrapped within the minute fat particles of the emulsion. After a time, the alkaline detergent began to act on the fat particles in the emulsion and saponified some of the fat, forming a soap, which then inactivated the bactericidal properties of the detergent by precipitating the ethyl cetab out of solution. As more fat particles were saponified, they released more entrapped bacteria to the solution, which was then no longer able to kill them.

To test this theory we made up a second set of detergent compositions that were designed to be emulsifying but nonsaponifying.

	No. 3	*No. 4*
Trisodium phosphate	50	50
Sodium bicarbonate	25	25
Tetrasodium pyrophosphate	25	25
Rodicide A[4]	6	
Nonionic wetting agent[5]		3
	106	103

Detergent solutions were made with formula no. 3 and formula no. 4 (1 oz per gal.) and tested as above, except that the washing time was extended to 2 hours to insure that no recontamination would occur because of slow saponification. The pH of both solutions was 10.0.

TABLE 1

Average number of bacteria per sq cm remaining on microscope slides

WASHING TIME IN MINUTES	NO. 1 pH 11.5	NO. 2 pH 11.5	NO. 3 pH 10.0	NO. 4 pH 10.0
1	8,000	17,500		
2	4,500	12,000	15,000	42,500
3	250	9,500		
4	65	8,500	0	1,500
5	0	5,000		
6			130*	3,000
8			25*	3,000
10	13,000	2,500	0	1,750
15			0	2,250
20			0	1,500
30			0	2,250
45			0	670
60			0	800
90			0	825
120			0	850

* Identified as *Bacillus subtilis*, not *Staphylococcus aureus*.

It was found that formula no. 4 (without rodicide A) is not bactericidal, although there was a drop in the bacterial count as the dishwashing time increased. Formula no. 3 (containing rodicide A) produced a very marked sharp drop in the bacterial counts. The slide removed after 4 minutes' washing time was sterile, and the slides removed after 6 and 8 minutes' washing time were free from *Staphylococcus aureus*, although they were contaminated with *Bacillus subtilis*. All slides removed after 8 minutes were sterile. Table 1 summarizes the results of both tests.

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[4] A product formulated by Rhodes Chemical Corporation, consisting of cetyl dimethyl ethyl ammonium bromide and an alkylated aryl polyether alcohol in aqueous-alcoholic solution.

[5] The same alkylated aryl polyether alcohol used in the two previous formulas.

The artificial soil was not sterilized before contamination with *Staphylococcus aureus*, and this organism was probably not the only contaminant, since *Bacillus subtilis* could easily have been introduced in preparing the soil. Complete removal of the soil from the slides without complete emulsification could account for the sterility of the slides in 4 minutes. As emulsification of the soil proceeded, *Staphylococcus aureus* and *Bacillus subtilis* could have been released from larger fat particles in which they were entrapped. The germicidal detergent, formula no. 3, killed *Staphylococcus aureus* more readily than *Bacillus subtilis*, which could account for the contamination with the latter organism of the slides removed after 6 and 8 minutes' washing time. Sporeforming organisms such as *Bacillus subtilis* are far more resistant to quaternary ammonium compounds than *Staphylococcus aureus* (Green and Birkeland, 1941; Hoogerheide, 1945; Du Bois and Dibblee, 1946).

SUMMARY

When cation-active agents are used as bactericides in detergent compositions, it is essential that the detergent be emulsifying but not saponifying. If the alkalinity of the detergent is sufficiently high to saponify fat, the resulting soap will inactivate the cation-active agent and render the solution completely ineffective germicidally.

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THE AMOUNT OF ENZYME INACTIVATION AT BACTERIOSTATIC AND BACTERICIDAL CONCENTRATIONS OF DISINFECTANTS

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The question whether the death of bacteria in sterilization is due to the destruction of energy-furnishing enzymes or to other changes in the cell has been tested experimentally by several investigators. In heat sterilization, Rahn and Barnes (1933) and Rahn and Schroeder (1941) found that enzymes are still active in cells that have lost the power to multiply. In chemical disinfection, the results are contradictory. While some find all or most enzymes still active when the treated cells are dead (e.g., Yudkin, 1937; Greig and Hoogerheide, 1941; Bucca, 1943), others claim that even at bacteriostatic concentrations the enzymes are injured (e.g., Dubos, 1939; Sevag, 1944).

A decision in this controversy can be obtained only if *all* energy-furnishing enzymes are taken into consideration. This was accomplished by working with a culture of *Escherichia coli* grown in a mineral solution with acetate as the only source of energy and of carbon, and ammonia as the only source of nitrogen. The medium described by Koser (1923) consisted of 0.5 per cent NaCl, 0.2 per cent $MgSO_4 \cdot 7H_2O$, 0.1 per cent $NH_4H_2PO_4$, 0.1 per cent K_2HPO_4, and 0.2 per cent CH_3COONa.

The cells were grown under continuous agitation at 37 C. They were centrifuged, washed in phosphate buffer (pH 6.8 to 6.9), recentrifuged, and suspended in new buffer. The cell concentration was standardized turbidimetrically.

The bacteriostatic concentrations were determined by observing the increase in turbidity of cultures in the acetate medium containing various amounts of each disinfectant. The bactericidal concentrations are the concentrations which kill *E. coli* grown in acetate medium within 5 to 10 minutes.

Energy is produced on this medium only by the oxidation of the acetate, involving either acetate dehydrogenase or oxidizing enzymes, and indirectly catalase. The activity of the dehydrogenase was determined by using Thunberg tubes containing 0.5 ml of 0.01 per cent aqueous methylene blue, 0.5 ml of 1 per cent sodium acetate, the necessary amount of poison, and distilled H_2O to make a total volume of 5 ml. One ml of a cell suspension was placed in the side arm and tipped in after evacuating the tubes and warming them to 37 C. The time required for 80 to 90 per cent decolorization of the methylene blue was determined, and the relative rate of enzyme activity was calculated by assuming that the rate is inversely proportional to the time required for decolorization. The rate is expressed in percentage of that of the cells without poison.

The catalase activity was determined by using the method of Sumner (1941). The poison was added to a buffered solution of H_2O_2, and the amount of undecomposed peroxide was determined at 3, 6, 9, and 12 minutes after the addition of a cell suspension. The rate of catalase action was measured by the amount of H_2O_2 decomposed in 3 minutes and expressed in percentage of the amount present at the beginning of the 3 minutes. The relative rate was expressed in percentage of the rate obtained when no disinfectant was present.

The O_2 uptake was measured with the Warburg respirometer, adding the desired amount of disinfectant from the side arm of the flask after the O_2 uptake of the cell suspension in acetate medium had reached a constant rate. The O_2 uptake was then observed for a period of 30 minutes and the relative rate calculated in percentage of the rate when no poison was added.

RESULTS

The results obtained with four characteristically different types of disinfectants are shown in figure 1. Their effect upon the cell enzymes is not at all uniform. Phenol at the retarding dose had not caused much damage to the enzymes. Most of the oxidase, but none of the dehydrogenase, was inactivated at complete inhibition of growth. With $HgCl_2$ the enzymes were not at all affected at complete inhibition of growth. The same was true with "ceepryn" (cetyl pyridinium chloride). Sodium azide, which at 1 per cent did not kill the bacteria, inactivated the catalase at extremely low concentrations, but did not affect the other two enzymes even at growth-inhibiting concentration. Table 1 gives the summary results for all disinfectants tested.

The rate of bacterial growth is in most cases measurably decreased at a concentration of disinfectants that does not decrease the rate of enzyme action in the cell. Even the concentrations that produce a complete cessation of growth do not, in most cases, retard enzyme action at all. The conclusion seems inevitable that the disinfectants prevent multiplication, not through inactivation of enzymes, but by some other reaction. This is true for $HgCl_2$, merthiolate, ceepryn, penicillin and sulfanilamide, and probably also for zephiran.

The picture at bactericidal concentrations is different. In the first five disinfectants of table 1, this concentration has inactivated practically all the dehydrogenase and oxidase. It is also of interest that the last four compounds, which do not kill bacteria, did not bring about such nearly quantitative inactivation of the two energy-furnishing enzymes.

It remained to be proved whether this inactivation of enzymes was reversible or permanent. This could be done with the oxidase in the Warburg respirometer by using double-armed flasks. First, the normal rate of respiration of the cells was established, then the disinfectant was added, and when its effect had been measured for 10 minutes, the antidote was added from the other arm. If thereby the O_2 uptake became normal, or at least improved, the enzyme inactivation was reversible.

With 10 ppm $HgCl_2$, the O_2 uptake of the cells was reduced to practically zero, and the addition of an equivalent amount of Na_2S did not produce any increase (figure 2). The oxidase had been irreversibly destroyed. With 50 ppm ceepryn, respiration was decreased. After the addition of duponol, an efficient antidote,

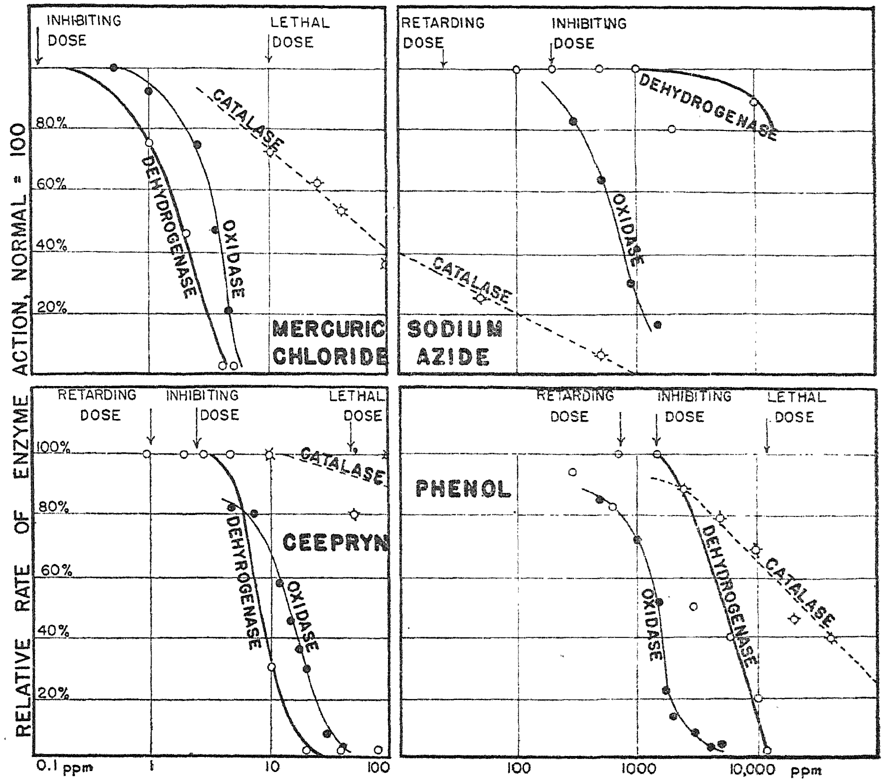

FIG. 1. EFFECT OF VARIOUS CONCENTRATIONS OF POISON ON THE ENERGY-FURNISHING ENZYMES OF E. COLI

TABLE 1

The effect of critical concentrations of disinfectants on the enzyme activity of Escherichia coli

(The numbers indicate the percentage of enzyme inactivation)

DISINFECTANT	RETARDATION OF GROWTH				INHIBITION OF GROWTH				DEATH			
	Concentration ppm	Dehydrogenase	Catalase	Oxidase	Concentration, ppm	Dehydrogenase	Catalase	Oxidase	Concentration, ppm	Dehydrogenase	Catalase	Oxidase
Phenol	750	0	9	20	1,500	0	10	48	12,000	96	38	96
$HgCl_2$	0.01	0	0	0	0.1	0	0	0	10	96	28	90
Merthiolate	0.001	0	0	0	0.1	0	0	0	125	96	8	90
Zephiran	2.5	0	0	0	10	0	0	30	40	96	0	90
Ceepryn	1.0	0	0	0	2.5	0	0	10	50	96	6	95
NaN_3	25	0	67	0	150	0	84	10	50,000*	30	100	100
KCN	25	0	97	68	500	0	97	90	50,000*	50	100	100
Penicillin	12.5	0	0	0	50	0	0	0	1,000*	0	0	0
Sulfanilamide	1,000	0	0	0	5,000	0	0	0	8,000*	0	0	20

* The concentrations did not kill in 10 minutes.

or of charcoal, which adsorbs ceepryn preferentially, the O_2 uptake proceeded at the same reduced rate. Here, too, the enzyme inactivation must be considered irreversible. The result with phenol was different. As no quickly reacting antidote against phenol could be found, the disinfectant was simply diluted with an equal amount of water. The recovery was almost complete, as may be seen from figure 2. However, the phenol concentration of 0.2 per cent used in this experiment is not lethal; 1.2 per cent phenol was needed to kill the bacteria in 10 minutes, and such cells, washed in buffer solution, after 10 minutes' exposure to 1.2 per cent phenol, showed no O_2 uptake at all. Thus, in all experiments, the concentration which kills *E. coli* in 10 minutes inactivates its dehydrogenase and oxidase irreversibly.

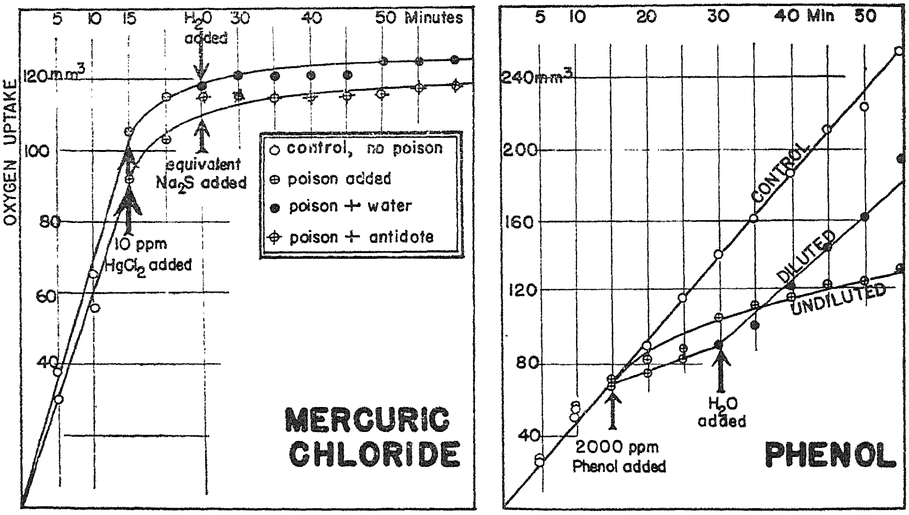

FIG. 2. EFFECT OF DILUTION AND OF ANTIDOTES ON THE RECOVERY OF POISONED ENZYMES OF E. COLI

It would be unwarranted, however, to conclude that death is due to irreversible inactivation of the enzymes. The alternative, that enzyme inactivation is the *result* of death rather than the *cause*, must also be considered. Hoffmann and Rahn (1944) and Rahn (1945) have shown that bacteriostasis and bactericidal action are due to two different chemical reactions. It has been shown above that bacteriostatic concentrations do not usually interfere with enzyme action. The reversible prevention of multiplication must therefore be due to the reaction of the disinfectant with some other cell constituent essential for multiplication, either with some step in synthesis or with some part of the cell division mechanism. Although this bacteriostatic reaction is reversible, the lethal reaction is not.

As a rule, the cells die slowly even at bacteriostatic concentrations, although their enzyme content is not noticeably impaired when exposed to these concentrations for a relatively short time. With slightly higher concentrations, however, a certain degree of parallelism between decrease in number of viable cells and enzyme destruction was found in the case of mercuric chloride.

To 100-ml portions of a 24-hour culture of *E. coli* in acetate medium were added 1, 2, and 3 ppm mercuric chloride at room temperature. The number of viable cells per ml and the oxygen uptake of the centrifuged cells resuspended in phosphate buffer were determined before addition of the poison, after a short exposure, and again after 24 hours' exposure.

One ppm mercuric chloride had no effect on the oxygen uptake or the number of surviving cells after 24 hours' exposure. Conflicting results were obtained when the cells were exposed to 2 ppm $HgCl_2$. In one experiment, a 72 per cent decrease in respiration and an 82 per cent decrease in the number of cells occurred after 5 hours, and after 24 hours a 70 per cent decrease in respiration accompanied a 90 per cent decrease in cells. The second experiment, however, showed an 83 per cent decrease in respiration with only a 7 per cent decrease in the number of cells after 4 hours. Three ppm $HgCl_2$ in 4 hours decreased the respiration 100 per cent and the number of living cells by 97 per cent. In each case, the addition of Na_2S to the cells produced no recovery or even partial recovery of the oxidase activity of the cells.

The sensitivity of the energy-producing mechanism to chemical disinfectants is very nearly the same as that of the growth-producing mechanism. The difference in sensitivity of the two mechanisms seems smaller with chemicals than with heat inactivation. There is no evidence that the inability of poisoned cells to multiply is brought about by lack of energy.

SUMMARY

The activity of all the energy-producing enzymes of *Escherichia coli* grown on acetate medium was determined in the presence of growth-retarding, growth-inhibiting, and lethal concentrations of poisons.

Retardation of growth in seven cases out of nine occurred at a concentration of poison that had little or no effect on the energy production. Concentrations that inhibit growth completely do not always inactivate or even retard the enzymes. At lethal concentrations, however, the enzymes are inactivated.

After removal of the poisons by dilution or antidotes, the enzyme activity of cells exposed to poisons was again determined. The dilution of a sublethal concentration of phenol resulted in reactivation of the inhibited oxidase. The inhibition of oxidase activity caused by lethal concentrations of ceepryn (cetyl pyridinium chloride) and mercuric chloride could not be reversed.

Experiments with concentrations between the bacteriostatic and the bactericidal dose of $HgCl_2$ showed that the enzyme mechanism is very nearly as sensitive as the multiplication mechanism.

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STUDIES ON HIGHLY SOLUBLE AZO SULFONAMIDES

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Since the early observations of Kellner (1936) and Fuller (1937), who demonstrated that *p*-aminobenzenesulfonamide (sulfanilamide) is formed in animal tissues when prontosil[1] or prontosil soluble[2] (neoprontosil[3]) is administered by mouth, and the studies by Domagk (1935), Colebrook and Kenney (1936), and Colebrook, Buttle, and O'Meara (1936), wherein they were unable to demonstrate an antibacterial effect on the part of the latter compounds *in vitro*, very little attention has been directed to similar studies on other azo sulfonamides. Inasmuch as several heterocyclic substituted sulfanilamide derivatives, namely, sulfathiazole, sulfapyridine, and sulfadiazine, have proved to be superior to sulfanilamide in their antibacterial effects against many organisms both *in vitro* and *in vivo*, it appeared worth while to compare the activity of these compounds with that of their azo sulfonamide counterparts.

An azo preparation with interesting properties, including the ability to inactivate lysozyme (Lawrence and Klingel, 1943), was prepared by coupling diazotized sulfathiazole with the symmetrical carbamide of 2R acid. The other soluble azo dyes were formed by coupling the diazotized parent sulfonamide compounds with sodium 1-hydroxy-7-acetylaminonaphthalene-3,6-disulfonate, hereafter described as "acetyl-2R acid." Diphenyl-sulfone-4,4′-disazo-acetyl-2R acid (a sulfone) and 6-sulfaquinoline-azo-acetyl-2R acid completed the series of drugs studied. The structural formulae and molecular weights of several of these compounds are given in figure 1.

The organisms against which the compounds were tested *in vitro* included *Staphylococcus aureus* 209, *Streptococcus pyogenes* C-203, a viridans streptococcus, types I, II, and III pneumococci, and members of the *Brucella* group.

IN VITRO METHODS

Studies on gram-positive cocci. Drug-broth solutions or suspensions of the compounds were prepared by adding the dry powders to 100-ml quantities of nutrient media contained in 250-ml cotton-plugged Erlenmeyer flasks. The test media, which also included a control lacking a drug, were autoclaved at 10 pounds for 10 minutes. Upon cooling, to each flask was added 1 ml of an 18-hour broth-diluted culture of one of the organisms.

The inoculated solutions were incubated at 37 C for 24 hours, after which time appropriate broth dilutions were made and 0.1-ml and 1.0-ml amounts were

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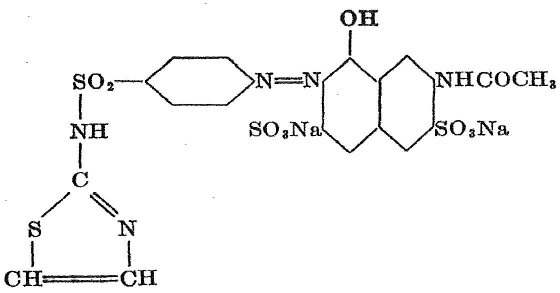
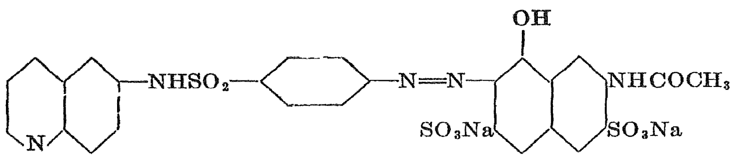
[1] 4-Sulfamyl-2′,4′-diaminobenzene hydrochloride.

[2] Sodium 1-hydroxy-2-(4 -sulfamylbenzene-1′-azo)-7-acetylaminonaphthalene-6-disulfonate.

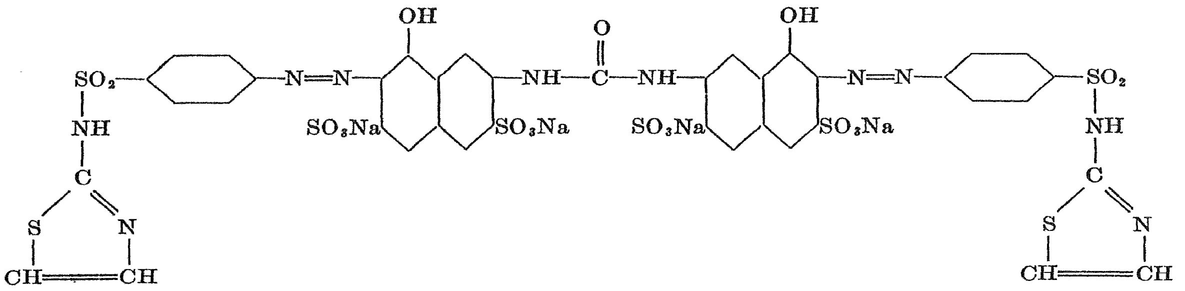
[3] The registered trademark of Winthrop Chemical Company, Inc.



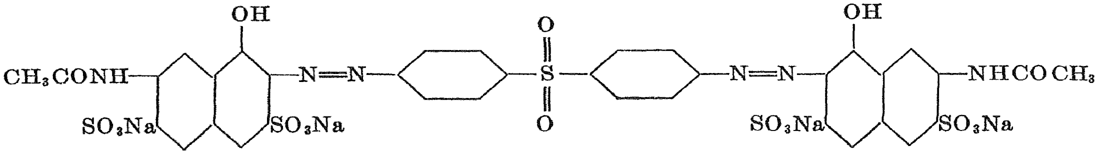
Sulfathiazole-azo-acetyl-2R acid
M.W. 671



6-Sulfaquinoline-azo-acetyl-2R acid
M.W. 715



Sulfathiazole-disazo-symmetrical carbamide-2R acid
M.W. 1,284



Diphenyl-sulfone-disazo-acetyl-2R acid
M.W. 1,080

FIG. 1 STRUCTURAL FORMULAE AND MOLECULAR WEIGHTS OF SEVERAL AZO SULFONAMIDES

placed in petri dishes, and melted and cooled (45 C) nutrient agar was added. The contents of the plates were mixed thoroughly by swirling, and the agar was allowed to solidify. The plates were incubated at 37 C for 48 hours, following which period the growing colonies were counted.

Beef extract broth (pH 6.8, bacto peptone) and agar were used in most of the tests against the staphylococcus and viridans streptococcus. However, additional studies were included in which veal infusion broth (pH 7.4, bacto proteose peptone) containing 1 per cent sterile horse serum was used. Veal infusion broth containing glucose, as well as serum, was used in the tests with pneumococci and *S. pyogenes*. Sterile defibrinated rabbit blood was added to the veal glucose agar to promote growth of the latter organisms and to facilitate counting of the colonies.

In table 1 are presented typical results of the *in vitro* effects of 1 per cent concentrations of the several azo derivatives and their sulfonamide analogues upon *S. aureus* and the viridans streptococcus. In order to obtain more highly concentrated drug-broth solutions of the parent substituted sulfonamides (sulfathiazole, sulfapyridine, and sulfadiazine) for testing the organisms mentioned, these compounds were used in the form of their sodium salts. The pH of each solution or mixture in beef extract broth, as determined by the glass electrode potentiometer, is given in table 1.

In general, the two azo derivatives of sulfathiazole and the parent compound gave comparable degrees of bacteriostatic activity upon *S. aureus* in beef extract medium. This relative effectiveness was reduced considerably in the presence of 1 per cent serum when veal infusion broth was used. Although there appeared to be an inhibitory effect of the azo derivatives of sulfapyridine and sulfadiazine upon the staphylococci, this action was inferior to that exhibited by the parent compounds in corresponding concentrations. A similar correlation in effectiveness was noted between sulfanilamide and its azo derivatives. Sulfathiazole-azo-acetyl-2R acid was found to be the most active compound against viridans streptococci. This drug was followed in order of activity by sulfapyridine and sulfathiazole. The remaining compounds gave but a suggestion of a similar effect upon the latter organism.

Since the pH values of 1 per cent sodium salt solutions of the parent analogues of several of the compounds are somewhat higher than the pH of solutions of the corresponding azo derivatives, it would appear that some of the variations in antibacterial activities (table 1) could be attributed to the differences in pH. Furthermore the possibility that a partial breakdown of the soluble azo dyes to their corresponding active parent analogues may occur during autoclaving also seemed to merit consideration. Both of these points were studied simultaneously by first sterilizing concentrated aqueous solutions of all drugs by heating them in a boiling water bath 2 to 3 minutes, following which time the appropriate amount of the solutions was added to the sterile nutrient broth to give a final concentration of 1 per cent of the sulfonamides. A complete series of drug-broth and control broth media lacking a drug was adjusted to pH 7.80 (1 per cent sodium sulfathiazole in broth) and another to pH 8.90 (1 per cent sodium sulfapyridine in broth) by the addition of sterile sodium hydroxide solution. With the use of *S. aureus* as the test organism in these studies, the differences in the degrees of activity of the compounds (table 2) were essentially the same as in table 1. In subsequent tests similar results were obtained when unheated, sterile, Seitz-filtered solutions were used.

TABLE 1

Effects, in vitro, of the compounds upon Staphylococcus aureus 209 and viridans streptococcus

COMPOUND	pH OF 1% DRUG-BROTH SOLUTIONS	1 PER CENT DRUG CONCENTRATIONS					
		Staphylococcus aureus				*Viridans streptococcus*	
		Beef extract broth		Veal broth 1% serum		Beef extract broth	
		Inoculum—organisms/ml					
		43	131	115	380	5,000	7,000
S	6.80	1.7*	3.6	0.26	90,000	23,000	29,000
S-Az	6.80	48,000	130,000	2,000	175,000	59,000	54,000
Na-ST	7.80	0.13	0.36	0.25	10,000	4,000	11,000
ST-Az	6.80	0.20	1.2	14	2,000	300	200
Na-SP	8.90†	4.8	15	0.50	60,000	2,000	3,000
SP-Az	6.85	53,000	25,000	10,000	75,000	69,000	67,000
Na-SD	7.30†	2,000	7,000	3,000	115,000	59,000	30,000
SD-Az	6.80	54,000	41,000	33,000	130,000	58,000	55,000
STC-Az	6.50	1.0	0.24	700	150,000	34,000	32,000
SQ-Az	6.70	2,000	27,000	1,000	115,000	86,000	86,000
D-Az	6.90	—	56,000	21,000	115,000	42,000	76,000
Control	6.90	118,000	335,000	125,000	180,000	84,000	76,000

Legend: S = sulfanilamide; S-Az = sulfanilamide-azo-acetyl-2R acid (prontosil soluble, "neoprontosil"); Na-ST = sodium sulfathiazole; ST-Az = sulfathiazole-azo-acetyl-2R acid; Na-SP = sodium sulfapyridine; SP-Az = sulfapyridine-azo-acetyl-2R acid; Na-SD = sodium sulfadiazine; SD-Az = sulfadiazine-azo-acetyl-2R acid; STC-Az = sulfathiazole-disazo-symmetrical-carbamide-2R acid; SQ-Az = 6-sulfaquinoline-azo-acetyl-2R acid; D-Az = diphenyl-sulfone-disazo-acetyl-2R acid.

* Figures, expressed in thousands (i.e., 1.7 = 1,700, etc.), represent number of cells per ml after 24 hours' incubation in broth + sulfa drug.

† Saturated solutions.

— = no test made.

The effects of 5×10^{-4} molar (M/2,000) concentrations of the several compounds upon types I, II, and III pneumococci and *S. pyogenes* C-203 are summarized in table 3. These data indicate that molar equivalents of the two azo derivatives of sulfathiazole, 6-sulfaquinoline-azo-acetyl-2R acid, and sulfathiazole itself were equally effective in inhibiting and, in some instances, in completely destroying the test organisms. Similar effects against the pneumococci were obtained with sulfapyridine, its corresponding azo derivatives, and diphenylsulfone-azo-acetyl-2R acid. However, a corresponding degree of activity upon *S. pyogenes* was not evidenced with the latter compounds. In general, sulfadiazine-azo-acetyl-2R acid was far inferior to sulfadiazine in inhibiting the growth of this organism. A still greater divergency in the extent of bacteriostatic activity was noted between sulfanilamide and its corresponding azo derivative.

TABLE 2

Effects, in vitro, of the compounds upon Staphylococcus aureus in broth adjusted to various pH values

COMPOUND	pH	pH UNADJUSTED	pH ADJUSTED TO 7.80	pH ADJUSTED TO 8.90
		Inoculum—organisms/ml		
		130	116	52
S	6.80	3.6	12	3.6
S-Az	6.80	130,000	182,000	3,000
Na-ST	7.80	0.36	10	1.8
ST-Az	6.80	1.2	6.1	0
Na-SP	8.90†	15	90	7.4
SP-Az	6.85	25,000	63,000	20,000
Na-SD	7.30†	7,000	3,000	110
SD-Az	6.80	41,000	75,000	65,000
STC-Az	6.50	0.24	40	0
SQ-Az	6.70	27,000	16,000	–
D-Az	6.90	56,000	156,000	–
Control	6.80	335,000	675,000	540,000

See legend under table 1.
0 = no growth.
† Saturated solutions.

Studies on Brucella organisms. A different test procedure was used in studies on the *Brucella* organisms. A concentration of 1.0 per cent of the drugs was prepared in tryptose phosphate broth containing 0.1 per cent agar. Serial dilutions were made in the same medium to include dilutions of 1:200, 1:400, 1:800, and 1:1,600. After autoclaving, to each tube containing 5 ml of test solution was added a 4-mm loopful of a 24-hour broth culture of either *Brucella abortus*, *Brucella melitensis*, or *Brucella suis*. The tubes were incubated at 37 C and observed for visible growth at time intervals of 24, 48, and 72 hours. Tubes show-

TABLE 3

Effects, in vitro, of the compounds upon pneumococci and Streptococcus pyogenes C-203

COMPOUND	5 × 10⁻⁴ MOLAR (M/2,000) DRUG CONCENTRATIONS							
	Pneumococcus types						*Streptococcus pyogenes* C-203	
	I		II		III			
	Inoculum—organisms/ml							
	30	400	3	10	10	80	11	293
S	132,000	20,000	2,500,000	15,000	0	4,000	117,000	565,000
S-Az	1,000,000	290,000	400	113,000	600	1,000,000	480,000	405,000
ST*	0.12	0.97	0	0	0	0	135	23,000
ST-Az	0.24	0.69	0	0	0	0.44	260	15,000
SP*	0.08	0.54	0	0	0	0.05	2,000	140,000
SP-Az	0.51	0.94	0	0	0.44	2,000	46,000	390,000
SD*	26	2	0	0.06	0	0.05	2,000	89,000
SD-Az	470,000	1,000	0	1,000	7,000	16,000	53,000	625,000
STC-Az	0.42	0.90	0	0	0	0.37	90	3,000
SQ-Az	0.03	0.29	0	0	0.18	1.7	6.0	3,000
D-Az	0.10	0.90	0	0	16	17,000	44,000	77,000
Control	2,000,000	485,000	930,000	226,000	222,000	250,000	230,000	610,000

See legend under table 1.

0 = no growth in plates inoculated with 0.1 ml of undiluted test mixture or dilutions thereof at the end of 24 and 48 hours' incubation of drug-broth-organism suspensions.

* Compounds were not used in the form of their sodium salts in this study.

ing no growth after 72 hours were tested for bactericidal action by transferring three 4-mm loopfuls to a tube of sterile tryptose phosphate agar broth. The results of this study are presented in table 4.

It will be observed that in general the azo derivatives compare in activity to equivalent weight concentrations of their respective parent sulfonamides in inhibiting the growth of the *Brucella* organisms. Broth dilutions of approximately 1:800 of all compounds proved to be bacteriostatic, and some were found to have a definite bactericidal effect against this group of gram-negative bacilli.

TABLE 4

Highest dilution of compounds showing antibacterial activity against Brucella organisms

COMPOUND	B. ABORTUS		B. MELITENSIS		B. SUIS	
	Bs	Bc	Bs	Bc	Bs	Bc
Na-S	1:400	1:400	1:800	1:400	1:1,600	1:200
S-Az	1:800	<1:100	1:400	<1:100	1:800	<1:100
Na-ST	1:1,600	1:800	1:1,600	1:400	1:800	1:400
ST-Az	1:1,600	1:200	1:800	1:200	1:200	<1:100
Na-SP*	1:400	1:400	1:800	1:400	1:1,600	1:200
SP-Az	1:400	<1:100	1:400	<1:100	1:400	<1:100
Na-SD	1:600	1:100	1:1,600	<1:100	1:800	<1:100
SD-Az	1:800	1:100	1:800	1:400	1:800	1:100
STC-Az	1:800	1:400	1:800	1:400	1:800	1:100
SQ-Az	1:800	<1:100	1:1,600	1:100	1:800	<1:100

See legend under table 1.
Na-S = sodium sulfanilamide.
Figures represent actual dilutions bacteriostatic (Bs) or bactericidal (Bc).
<1:100 = concentrations greater than 1:100 not tested.

IN VIVO METHODS

Chemotherapy of experimental streptococcal and pneumococcal infections in mice. Male albino mice weighing approximately 20 grams were inoculated intraperitoneally with 0.3 ml of a saline suspension of an 18-hour, veal glucose serum broth culture of either *S. pyogenes* C-203 or type II pneumococcus. The animals were medicated by means of a stomach tube 1½, 7, 12, 24, 32, and 48 hours after infection, and once daily thereafter until death occurred or up to and including the tenth day. The preparations were administered as a suspension or solution in 0.25 to 0.5 ml of milk. The results of studies in which sulfapyridine and its corresponding acetyl-2R acid derivative were used are presented in table 5.

It is apparent from the data given in table 5 that the sulfapyridine component of the azo sulfonamide is the portion of the molecule responsible for prolonging life or protecting the mice from experimental streptococcal and pneumococcal infections; when gram equivalents of the two compounds are used, the parent sulfonamide is more effective in protecting the animals.

Chemotherapy of experimental Brucella abortus infections in mice. Kolmer (1940) tested five sulfonamides in the treatment of acute brucellosis in mice infected with *B. abortus* and obtained favorable therapeutic results with all of them. The compounds included sulfanilamide and its azo derivative neoprontosil. Both compounds appeared to be about equally effective in increasing the survival rate of infected mice. In our study the chemotherapeutic value of sulfadiazine and its azo derivative was compared in a similar brucellosis infection in mice.

TABLE 5

Chemotherapy of beta hemolytic streptococcus and type II pneumococcus infections in mice

ORGANISM AND COMPOUND	NUMBER MICE	DOSE		SURVIVAL IN HR		SURVIVAL: 10-DAY % OF SERIES
		Single	Total	Range	Median	
		mg/kg	*g/kg*			
Beta hemolytic streptococcus C-203						
Sulfapyridine	10	25	0.35	80–164	96	70
	10	100	1.40			100
	10	250	3.50			100
Sulfapyridine azo-acetyl-2R acid	10	25	0.35	21–240	53	20
	10	100	1.40	44–72	60	70
	10	250	3.50	220	220	90
Control—no drug	10			10–24	14	0
Type II pneumococcus						
Sulfapyridine	10	250	3.50	50–94	74	20
	10	500	7.00	48–196	78	20
	15	1,000	14.00	76–160	116	40
Sulfapyridine azo-acetyl-2R acid	10	1,000	14.00	66–116	71	10
	10	2,000	28.00	68–240	91	40
Control—no drug	10			25–71	40	0

Male albino mice weighing approximately 20 grams were injected intraperitoneally with 0.2 ml of a saline suspension of a 24-hour tryptose phosphate agar slant culture of *B. abortus*. With the exception of one group of 10 mice, the animals were medicated by means of a stomach tube immediately after injection, 4 hours later, and twice each day thereafter for a total period of 14 days. The other group of mice was maintained on the same medication schedule, but the drug was administered by the subcutaneous route. The observation period continued for a total of 18 days. The drugs were administered as an aqueous solution or suspension.

Kolmer obtained his best therapeutic results against *B. abortus* infections in mice with the sodium formaldehyde sulfoxylate derivative of sulfanilamide, which produced a survival of 56.2 per cent. In our tests sulfadiazine therapy showed a survival of 70 to 75 per cent, and the azo derivative of sulfadiazine caused a survival of 80 per cent. Oral medication of the azo compound appeared to be slightly more effective than subcutaneous administration.

TABLE 6

Chemotherapy of Brucella abortus infections in mice

COMPOUND	NUMBER MICE	DOSE		SURVIVAL—HOURS		SURVIVAL—18 DAYS % OF SERIES
		Single	Total	Range	Median	
		mg/kg	*mg/mouse*			
Sulfadiazine	20	100 oral	56	144–168	156	70.0
	20	50 oral	28	236	236	75.0
Sulfadiazine azo-acetyl-2R acid	30	100 oral	56	236	236	80.0
	20	50 oral	28	96–120	108	80.0
	10	100 subq.	56	168	168	70.0
Control—no drug	30			96	96	23.3

TABLE 7

Effect of azo sulfonamides upon bacterial flora (E. coli) of intestinal tracts of mice

TEST DAY	DESOXYCHOLATE AGAR PLATE (E. COLI) COUNT GROUP NO. (REPRESENTING 5 MICE EACH)			
	1	2	3	4
	Normal diet			
3rd	I	H	I	I
	Sulfathiazole-azo-acetyl started on 3rd day		Sulfanilamide-azo-acetyl started on 3rd day	
5th	20	20	I	I
7th	0	0	I	I
	Normal diet begun on 7th day			
9th	I	I	I	I
13th	I	T	I	I
			Sulfathiazole-azo-acetyl started on 13th day	
16th	—	—	0	0
19th	—	—	0	0
	Normal diet begun on 19th day			
22nd	—	—	T	I
26th	—	—	I	I

Legend: I = innumerable (too many to count); T = thousands; H = hundreds; figures represent actual number of lactose-fermenting colonies in plate; 0 = no lactose-fermenting colonies observed; — = no test made.

Effects of azo sulfonamides upon intestinal coliform organisms in mice. Marshall *et al.* (1940) noted that the incorporation of sulfanilylguanidine in the diet of normal mice would cause a distinct reduction in the usual number of coliform organisms in the stools. Subsequent studies from our laboratories (Lawrence and Sprague, 1941) confirmed the observations of Marshall and his coworkers on sulfanilylguanidine. We noted, in addition, that sulfathiazole and sulfapyridine also caused a significant reduction of the number of coliform organisms normally present in the intestinal excreta of mice. Sulfanilamide, on the other hand, proved to be entirely ineffective in lowering the fecal *Escherichia coli* count of the animals.

In the present study several of the azo sulfonamides were tested for their ability to reduce the *E. coli* population in the stools of mice. A slight modification from the procedure used previously was made in the method for determining the effects of the compounds upon coliform organisms. Instead of plating fecal suspensions from each individual animal, approximately equal weights of stool specimens from each of a group of five animals were pooled, emulsified, and then plated in desoxycholate agar. Otherwise, the technique for estimating antibacterial actions of 1 per cent drug diets was the same as described before. Table 7 presents the results of a typical experiment using the azo derivatives of sulfanilamide and sulfathiazole. Note should be made of the observation that the azo compounds were excreted in the urine as well as in the feces as evidenced by the red pigmentation of the excreta.

From the data given in table 7 it will be noted that sulfathiazole azo-acetyl-2R acid caused a distinct lowering or complete disappearance of the *E. coli* flora of the medicated animals. The same attribute, however, was entirely lacking when the azo derivative of sulfanilamide was used. The data, furthermore, reveal that the same group of animals which failed to respond to the latter compound would, nevertheless, show a lowering of coliform organisms when placed on an azo sulfathiazole diet. Sulfadiazine azo-acetyl-2R acid gave results comparable to those obtained with the sulfathiazole derivative, whereas the azo analogue of sulfapyridine was somewhat less effective.

DISCUSSION

The limited solubility of the commonly known sulfonamides in aqueous solutions has resulted in the use of the sodium salts of the compounds when relatively high concentrations of the drugs are desired. The principal objection to the use of the sodium sulfonamides in wound therapy, irrigations, or in topical application has been the strong alkaline property of the compounds, with a pH of 10 or 11, which is caustic to tissues. Fletcher (1941) irrigated the antrums of a patient with sodium sulfathiazole and noted that the lining membrane was completely "charred" and destroyed. He attributed this toxic effect to the caustic action of the drug.

Adams (1943) recommended the use of sulfanilamide solutions in preference to sulfathiazole, sulfadiazine, etc., in disinfecting infected pulp and periapical lesions. He concluded from his studies that the neutral soluble derivatives of the latter sulfonamides were objectionable, for they are so nearly insoluble that they persist in the tissues long enough to become foreign body irritants.

Wein *et al.* (1945) have recently described derivatives of sulfathiazole and sulfapyridine that are highly soluble, yet their local toxicity and irritant properties are less than those of the sodium salts of the sulfonamides. The compounds, "soluthiazole" and "solupyridine" are disodium cinnamylidene dibisulfite derivatives of the corresponding parent sulfonamides. They were found to be as active as the sodium salts of sulfathiazole and sulfapyridine in streptococcal and pneumococcal infections in mice. High concentrations of aqueous solutions of the solusulfonamides gave pH values of about 8.0.

Ten per cent solutions of many of the azo sulfonamides tested in our studies are readily obtainable by adding the powders to cold distilled water. In the concentration given, the sulfanilamide, sulfadiazine, sulfathiazole, and the sulfathiazole-symmetrical-carbamide derivatives give pH values of 5.3, 6.0, 3.7, and 7.1, respectively. The sulfapyridine and quinoline compounds are somewhat less soluble than the latter azo derivatives. One per cent aqueous solutions of the former two dyes have pH values of 6.70.

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SUMMARY

In vitro studies on several azo sulfonamides indicate that some of these soluble dyes compare favorably with their parent analogues in inhibiting the growth of gram-positive cocci. The azo derivatives of sulfathiazole appeared to be the most effective dye compounds tested with respect to their bacteriostatic and possible bactericidal actions on several of the organisms studied. Following, in order of diminishing activity, were the azo derivatives of sulfapyridine, sulfadiazine, and sulfanilamide.

Based on the assumption that the azo compounds owe their activity to the liberation in the animal body of the parent sulfonamides, evidence is presented to show that some of the azo derivatives are effective in protecting mice experimentally infected with pneumococci, streptococci, or *Brucella abortus*.

Incorporation of the heterocyclic, nonalkaline azo sulfonamides in the diet of normal mice resulted in a definite reduction in the coliform flora of their intestinal excreta.

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THE VIABILITY OF DRIED SKIM-MILK CULTURES OF LACTOBACILLUS BULGARICUS AS AFFECTED BY THE TEMPERATURE OF RECONSTITUTION

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In the bacteriological examination of dried milk products considerable attention has been given to the elimination of particles of undissolved milk that might interfere with the counting of colonies on agar plates. Roller-dried milk and dried acid milk products have presented the greatest difficulty in this respect, but spray-dried milk has been relatively easy to reconstitute into solution. Warm water (Macy, 1928; Downs *et al.*, 1932; Standard Methods for the Examination of Dairy Products, 1941) and N/10 or N/5 lithium hydroxide (Prickett and Miller, 1932, 1933; Prickett, 1939; Standard Methods for the Examination of Dairy Products, 1941) have usually been used to solubilize the more insoluble powders during reconstitution. Indeed, more attention has been given to the foregoing aspects than to the effect of the various reconstituting methods upon the bacterial cells themselves.

Hiscox (1945) demonstrated that spray-dried milk powders gave consistently much higher bacterial counts when reconstituted in Ringer's solution at 50 C than with Ringer's solution at 18 to 22 C. The increased count was obtained only when the solution at 50 C was added to the dry milk and not when the dry product was reconstituted at 18 to 22 C and then warmed to 50 C. It was suggested that the heat had a stimulating effect on the dry bacterial cells. Higginbottom (1945) also observed the heat effect noted by Hiscox but believed that the reconstitution at 50 C of spray-dried skim milk caused a change in the colloidal condition of the protein suspension, and resulted in a breaking up of bacterial clumps.

In the present work we have studied the effect of the temperature of reconstitution on the colony count of spray-dried skim-milk cultures of *Lactobacillus bulgaricus*, and have attempted to show the manner in which the heat affects the colony count of the product.

EXPERIMENTAL

The culture used in these studies was *Lactobacillus bulgaricus* no. LB2, a culture maintained in this laboratory for a number of years. On agar media, as described later in the paper, it formed a characteristic irregular and somewhat fuzzy type of colony about 2 mm in diameter, and conformed to the general characteristics of this species (Bergey *et al.*, 1939). Skim-milk cultures of this organism were spray-dried, and, in order to facilitate handling, the dried culture was usually blended with dried whey. Some of the work also was with tablets[1] prepared from the blended culture.

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[1] **We wish to thank Hynson, Westcott, and Dunning, Inc., Baltimore, Maryland, for supplying us with these tablets.**

The cultures were examined for viable counts by weighing 11 g or 1 g into a dry, wide-mouth 8-oz jar, equipped with a screw top containing a rubber gasket. In the examination of the tablet cultures, the tablets were ground in a sterile mortar before reconstitution. Then 99 ml of reconstituting fluid were quickly added, and the mixture was immediately shaken vigorously 50 times over a distance of about 1 foot. It was allowed to stand 5 minutes at the temperature of reconstitution, a water bath being used to maintain temperatures above that of room temperature during the holding period. This was followed by a second shaking of 50 times, and the reconstituted sample was diluted in water blanks at room temperature and plated. Dilutions were prepared so that 1 ml of a given dilution was in each culture dish. Counts were made with duplicate or triplicate plates. About 15 ml of agar medium were used per plate for mixing with the sample, and after solidification a second portion of about 10 ml was used as an overlay. Plates were incubated 2 to 3 days at 37 C. No increase in counts was obtained when incubation was for longer times.

In counting the viable cells of *L. bulgaricus* we usually used special whey agar, according to the formula of Farr (1935) with several minor changes. This medium is prepared as follows:

1. Preparation of special whey. Heat fresh skim milk at 18 to 20 pounds' steam pressure for 3 hours. Filter through no. 12 Whatman filter paper. Sterilize in convenient amounts.
2. Preparation of the agar medium. Formula:

Special whey	50.0 ml
Agar-agar	1.5 g
Gelatin	0.3 g
Proteose peptone (bacto)	0.5 g
Glucose	0.3 g
Sucrose	0.3 g
Distilled water	50.0 ml

Mix all ingredients except the agar and heat 10 minutes in the steamer; adjust to pH 6.6; filter through no. 12 Whatman filter paper; add an equal volume of 3 per cent melted and filtered agar; sterilize 20 minutes at 15 pounds' steam pressure.

In addition we have used BBL trypticase sugar agar as formulated by McLaughlin (1946) with essentially the same results.

RESULTS

The reconstitution of different forms of the spray-dried *L. bulgaricus* culture in water at 50 C, compared with reconstitution in water at room temperature (21 to 25 C), showed that the former temperature always gave a higher colony count (table 1). Although there were some variations in the increase in counts of the culture at a given age, the older cultures showed much higher increases than the freshly dried ones. Reconstituting fluid tempered to 37 C and 43 C also gave higher colony counts than were obtained when reconstitution was at 21 to 25 C, and the counts were essentially those obtained by using 50 C reconstitution.

Since warmed water is generally used for reconstitution to aid in the solubility of milk powders, it might be expected that the increased counts obtained by the use of water at 50 C were caused by more bacterial cells being liberated from milk particles as a result of the increased solubility of the powder at 50 C. If this were the effect of the heat, the use of nontoxic solutions that would dissolve the milk particles at room temperature should thereby increase the colony count by liberating any entrapped bacteria. In a survey for such a fluid it was found that 0.5 per cent sodium citrate at 50 C and at 21 to 25 C effected complete solution of the various forms of the dried skim-milk culture of *L. bulgaricus*, and did not show toxic effects when reconstituting this culture. Other reconstituting fluids tried were sterile skim milk, which gave results comparable to 0.5 per cent sodium citrate; 0.05 per cent nacconol, which was slightly toxic; and N/10 lithium hydroxide, which was very toxic. The results (tables 2, 3, and 4) showed that, although the dry culture was in solution with the 0.5 per cent sodium citrate, reconstitution at 50 C with the same fluid resulted in obtaining a much higher colony count.

TABLE 1

Increase in the colony count of dried skim-milk cultures of L. bulgaricus (of different ages) when reconstituted in water at 50 C compared with water at room temperature (21 to 25 C)

CULTURE	THE RATIO OF THE COLONY COUNT PER G AT 50 C TO THE COLONY COUNT PER G AT 21 TO 25 C				
	Fresh (1 to 4 days)	3 months	6 months	12 months	15 msthon
Blended 54	1.78				
Spray-dried 56	1.52				
Blended 53	1.43	1.77			
Tablet 53	1.51	1.89			
Spray-dried 53	1.72		3.94		
Tablet 52		2.06	1.38		
Blended 52		2.16	4.08		
Blended 49			4.78		
Tablet 49			2.16		
Blended 50			2.16		
Tablet 50			4.94		
Blended 51			3.13		
Blended 46				4.47	
Tablet 47				2.87	
Blended 48				3.79	
Spray-dried 48					5.93
Blended 47				4.09	4.40
Average	1.59	1.97	3.32	3.81	5.17

Microscopic examination of reconstituted spry-dried cultures confirmed the fact that reconstitution in water at 50 C results in solution of more skim-milk particles than when water at room temperature is used. The use of 0.5 per cent

TABLE 2

The effect of reconstituting dried L. bulgaricus at room temperature and then warming to 50 C

CULTURE	RECONSTITUTING FLUID	RECONSTITUTED AT R. T. AND HELD AT R. T.	RECONSTITUTED AT R. T. AND HEATED TO 50 C	RECONSTITUTED AT 50 C AND HELD AT 50 C
		millions/g	*millions/g*	*millions/g*
Blended 47	Distilled water	2.31	2.18*	5.5
Blended 50	Distilled water	45.0	34.0†	95.0
Blended 47	Distilled water	1.32	1.64†	5.80
Spray-dried 48	0.5% sodium citrate	16.4	18.8‡	64.0

* Heated to 50 C in 10 minutes; held at 50 C 5 minutes.

† Heated to 50 C in 8 to 10 minutes.

‡ Heated to 45 C; held in 50 C bath 5 minutes.

TABLE 3

The effect of using an intermediate dilution for heat-treating L. bulgaricus after reconstitution of the dry culture at room temperature

CULTURE	RECONSTITUTING FLUID	RECONSTITUTED AT ROOM TEMPERATURE (21 TO 25 C)	RECONSTITUTED AT ROOM TEMPERATURE; NEXT DILUTION AT 50 C	RECONSTITUTED 50 C
		millions/g	*millions/g*	*millions/g*
Spray-dried 48	0.5% sodium citrate	16.4	15.3	64.0
Blended 47	Distilled water	1.32	1.24	5.80
Spray-dried 48	Distilled water	7.7	7.8	52.0

TABLE 4

The effect of reconstituting dried L. bulgaricus at 50 C on the colony count and activity in skim milk compared with reconstitution at 21 to 25 C

CULTURE	METHOD OF RECONSTITUTION	COLONY COUNT OF DRY CULTURE	ACIDITY IN SKIM MILK			
			20 hr	24 hr	29 hr	49 hr
		millions/g	%	%	%	%
Blended 47 (Expt. 1)	Water at 21 to 25 C	1.46	0.33	0.47*	—*	—*
	Water at 50 C	6.55	0.60	0.93	1.35	2.04
	0.5% sodium citrate at 21 to 25 C	2.09	0.53	0.81	1.16	1.85
	0.5% sodium citrate at 50 C	6.35	0.61	0.94	1.40	2.05
Blended 47 (Expt. 2)	Water at 21 to 25 C	2.35	0.37	0.72	0.80	–
	Water at 50 C	4.40	0.40	0.90	1.20	–
	Skim milk at 21 to 25 C	3.10	0.39	0.81	1.00	–
	Skim milk at 50 C	7.60	0.45	0.99	1.30	–

* Abnormal fermentation.

sodium citrate, either at 50 C or room temperature, effected complete solution of all particles of skim milk. There was no difference in the cell count, however, as determined by counting a suitable dilution in a blood counting chamber, whether the culture was reconstituted at 50 C or at room temperature, and there was no difference in the clumping of individual cells, most of them existing singly or in pairs, particularly in the 0.5 per cent sodium citrate in which solution was complete. Centrifugation tests on the reconstituted powder showed essentially the same effects of the heat and sodium citrate on the solubility of the cultures, although it was not felt that it measured these effects as accurately as the microscopic examinations. These results indicated that the heat had a stimulatory or activating influence on a very large number of the dry cells when the culture was reconstituted at 50 C.

The effect of the heat on increasing the colony count was evident only when the original dry culture was reconstituted with fluid tempered to 50 C. Thus it was not possible to obtain the increased count by reconstituting the dry culture in fluid at room temperature and then warming the mixture, using mild stirring, to 50 C (table 2). The culture "spray-dried 48" was reconstituted at room temperature and then heated to 45 C before the holding period in the 50 C bath was started, since it was observed that adding 99 ml of fluid at 50 C to a 1-g sample caused the temperature to drop to 45 C. In these experiments reconstitution at room temperature followed by warming to 50 C gave colony counts of the same magnitude as those reconstituted and held at room temperature. Reconstitution at 50 C gave the usual large increase in counts. These data indicated that the heat must be applied as a shock to the dried cultures in order for it to have its effect in stimulating or activating the cells. These observations are in accord with those of Hiscox (1945) in her study on the plate count of milk powders.

TABLE 5

A comparison of the effects of the reconstitution temperature on the colony counts of freeze-dried and spray-dried skim-milk cultures of L. bulgaricus

CULTURE	RECONSTITUTING FLUID	RECONSTITUTED AT ROOM TEMPERATURE	RECONSTITUTED AT 50 C
		millions/g	*millions/g*
Freeze-dried 56	Distilled water	2,070	840
Freeze-dried 56	0.5% sodium citrate	1,970	800
Spray-dried 56	Distilled water	910	1,380
Spray-dried 56	0.5% sodium citrate	780	1,270

Further evidence that the heat must be applied to the dry culture at the time the cells are being rehydrated was gained by preparing an intermediate dilution in water at 50 C (table 3). In these experiments the dry culture was reconstituted in water or 0.5 per cent sodium citrate at room temperature in the usual manner. The next dilution was then made in water at 50 C and held 5 minutes at 50 C before the next dilutions in water at room temperature were prepared. The data so obtained showed that this treatment gave no increase in count over the preparation of all dilutions at room temperature. Reconstitution at 50 C, on the other hand, gave the usual increase in colony counts.

The activity of dry cultures after reconstitution at 50 C and room temperature was measured by inoculating 1 ml of the reconstituted culture (11 g blended culture in 99 ml fluid) into 99 ml of sterile skim milk, followed by incubation of the milk at 37 C. The titratable acidity of the milk cultures was then measured at various intervals (table 4). As would be expected, the cultures reconstituted at 50 C developed acidity more rapidly than those reconstituted at room temperature, a fact which indicated that the cells activated by the heat were physiologically active in the skim milk as well as in the agar plating medium.

Part of one batch of skim-milk culture of *L. bulgaricus* was freeze-dried the same day that the main part was spray-dried, giving a culture of the same age but dried by the two methods. The spray-dried culture gave a substantially higher count when reconstituted at 50 C than when reconstituted at room temperature. The freeze-dried culture, however, when reconstituted at 50 C gave a lower count than when reconstituted at room temperature (table 5). Reconstituting the freeze-dried powder with ice water failed to give any better colony count than reconstitution with water at room temperature.

DISCUSSION

The results of this study have shown that heat has an activating influence on the dry cells of a spray-dried skim-milk culture of *L. bulgaricus* when these cells are reconstituted. That the heat has a deleterious effect on a freeze-dried skim-milk preparation of the same culture indicates a marked difference in the cells when dried by the two methods. No explanation of the differences in the cells is presented at this time. It appears, however, that the heat applied in the spray-drying process leaves many of the cells in a condition in which they can be activated by rehydration in the presence of heat. What vital part of the cell—whether physical or enzymatic—responds to this treatment remains to be determined.

The surprising results are that heat applied, after the dry cells have once been reconstituted in cold liquid, does not have the activating effect observed when the cells are reconstituted in warmed liquid. Thus, whatever is activated by the heat is so affected by the shock obtained during reconstitution in warmed liquid. Heating the dry culture to 50 C did not cause any activation, but an actual destruction of some of the cells.

It should be noted that Allen (1923) observed that a liquid culture of a micrococcus after being heated at 145 F for 30 minutes reduced its generation time from 5 hours, 25 minutes to 2 hours, 7 minutes after the heating. Similarly, Voss and Frazier (1945), using a skim-milk culture of *Streptococcus thermophilus*, found that the generation time of this culture was reduced from 40 and 42 minutes to 24 and 29 minutes, respectively, after heating at 60 C 30 minutes. Although these data were secured with liquid cultures and are scarcely applicable to dried cultures, they do show that heat can have a definite stimulatory effect on the reproductive mechanism of cells of certain species. Rather than causing an acceleration in the rate of reproduction, the heat apparently only activated into normal growth a large number of the spray-dried cells of *L. bulgaricus*.

SUMMARY AND CONCLUSIONS

In spray-dried skim-milk cultures of *Lactobacillus bulgaricus* a large number of cells that failed to grow when the temperature of the reconstituting fluid was 21 to 25 C were activated sufficiently to produce normal growth when the temperature of the reconstituting fluid was 37 to 50 C. When the culture was reconstituted at 21 to 25 C and warmed to 50 C, the activating effect of the heat was not obtained.

The increase in the colony count resulting from reconstitution at 50 C over reconstitution at 21 to 25 C could not be explained by an increase in the solubility of the powder, nor an increase in the dispersion of the cells.

Reconstitution of dried *L. bulgaricus* at 50 C with subsequent inoculation into skim milk showed greater activity in the skim milk, particularly in the early stages of growth, than was obtained when the culture was reconstituted at 21 to 25 C.

Freeze-drying part of a skim-milk culture that was also spray-dried showed that cells in the freeze-dried culture were not only not activated by reconstitution at 50 C but that this temperature actually was lethal to many of the cells. This suggested a physiological difference between freeze-dried and spray-dried cells of *L. bulgaricus*, since the latter were markedly activated by heat.

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FATTY MATERIAL IN BACTERIA AND FUNGI REVEALED BY STAINING DRIED, FIXED SLIDE PREPARATIONS[1]

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Following Hartman, who first suggested the use of Sudan black B, in place of red Sudans, as a bacterial fat stain (Hartman, 1940), Burdon, Stokes, and Kimbrough (1942*a*) confirmed the greater value of this dye and modified the procedure for demonstrating intracellular fatty material in bacteria by preparing, from suspensions of the organisms in alcoholic Sudan black B solution, *dried films* counterstained with safranine. Previously it had been thought that dried, fixed films were entirely unsuitable for fat stains (Lewis, 1941).

These permanent films were regarded as an obvious improvement over the wet preparations used by earlier workers, and they were shown to be of practical aid in the classification of aerobic sporeforming bacilli (Burdon, Stokes, and Kimbrough, 1942*b*), but the staining method still had a number of undesirable features.

Further experimentation has resulted in the development of the much superior procedure to be described here. The new method is not only simpler, requiring no more effort than a gram stain, but it is also far more rewarding, for the films now reveal clearly intracellular lipid matter that previously has not been seen or even suspected. The improved stain has increased differential value. Moreover, its application to various bacteria has resulted in certain general findings of unusual interest.

SUDAN BLACK B FAT STAIN FOR FIXED PREPARATIONS

Technique. 1. Prepare the film, let it dry thoroughly in the air, and fix it by heat in the usual way. (Chemical fixation has no special advantages and may result in some loss of demonstrable lipid.) 2. Flood the entire slide with Sudan black solution (0.3 g of the powdered stain[2] in 100 ml of 70 per cent ethyl aclohol), and allow the slide to remain undisturbed at room temperature for 5 to 15 minutes. (A staining period of less than 5 minutes will often suffice, but the intracellular lipid is colored somewhat more intensely when the staining is continued for 5 minutes or longer. No further staining apparently occurs after the solution precipitates and turns a greenish or brownish color, but no harm is done if the stain is allowed to dry completely over the film. 3. Drain off excess stain and blot the slide thoroughly dry. 4. Clear the slide with cp xylol by dipping it in and out of the solvent in a Coplin jar or by adding xylol from a dropping bottle. Blot the cleared slide dry. 5. Counterstain with safranine (0.5 per cent aqueous solution) for 5 to 10 seconds (for ordinary bacteria or fungi), or with dilute carbol fuchsin (Ziehl's carbol fuchsin diluted 1:10 with distilled water) for 1 to 3 minutes (for acid-fast organisms). (Overstaining with the counterstain must be avoided). 6. Wash in water, blot, and dry the slide.

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[1] Presented, in part, before the 45th General Meeting of the Society of American Bacteriologists at New York, N. Y., May 3, 1944.

[2] The original, imported Sudan black B dye is not available commercially at the time of writing. But recently the National Aniline Division, Allied Chemical and Dye Corporation, 40 Rector Street, New York 6, N. Y., has developed a duplicate of this stain, and the company promises to have a supply of the American-made Sudan black B on the market shortly. In my hands this new product has given results equal to those obtained with the imported dye. The writer is indebted to Dr. H. J. Conn, president of the Biological Stain Commission, for his kindness in making various dye samples available for comparative tests.

Comment. After the bulk of the dye has been dissolved, the Sudan black B solution should be thoroughly shaken at intervals, then allowed to stand overnight before use. It remains good for several months at room temperature, provided it is kept in a well-stoppered, chemically clean container.

The entire slide is flooded with the staining solution to prevent the too rapid evaporation that otherwise occurs. (For reasons not entirely understood, the staining is generally unsatisfactory when slides are immersed in the Sudan black B solution in a Coplin jar.) Since the cellular lipid in most organisms takes up the characteristic blue-black color almost at once, it is possible to complete the whole fat-staining procedure within a minute or two if desired. On the other hand, if the technician is occupied with other tasks, the stain may simply be allowed to dry on the slide; then the clearing with xylol and counterstaining may be carried out later at a more convenient time. If the Sudan black B solution is allowed to stand on the slide for about 15 minutes and is then set afire by applying the Bunsen flame to the fluid, followed by blotting and xylol-clearing as usual, the intracellular fat in some organisms (e.g., gonococci) is brought out more clearly. Ordinarily, this step is unnecessary.

Examination of the cleared preparation without counterstaining is sometimes interesting and revealing. Care should be taken to avoid obscuring very tiny fat droplets by too strong a counterstain. Films must be examined with the oil immersion lens under critical illumination. To discern the smallest lipoid particles the observer must have a good sense for the color distinction between the blue-black or blue-gray of the fat droplets and the pink of the counterstain.

RESULTS OF APPLYING THE STAIN TO VARIOUS SPECIES OF BACTERIA AND FUNGI

Methods. The stain has now been applied to films from cultures of virtually all the chief species of bacteria, and of many fungi. In order to obtain a general picture of the occurrence of demonstrable intracellular lipid in these organisms and to permit a comparison between species in this respect, films were made from all cultures at approximately the same stages of growth, i.e., in early maturity (12 to 24 hours old). For the purpose of this preliminary survey, the organisms were grown on the media customarily employed for routine cultures of the species concerned, such as plain extract or infusion agar, potato slants, blood or ascitic fluid agar (for pneumococci, streptococci, gonococci, etc.), Loeffler's serum slants (for the *Corynebacterium* group), blood agar slants or thioglycolate media (for anaerobes), coagulated egg media and glycerine agar (for acid-fast bacilli), and Sabouraud's medium (for fungi). The majority of the films were made from stock cultures, under conditions permitting accurate comparisons. A smaller number of observations were made on preparations from primary (mixed) cultures, derived from the dust or from the throat, skin, etc., of human beings, and on direct films from the human mouth or from sputum, pus, and the like.

General findings. The films showed that fatty material staining with Sudan black B is present, often in conspicuous amounts, in the great majority of microorganisms, whether aerobic or anaerobic, saprophytic or parasitic, pathogenic or nonpathogenic, and that it is to be seen in a considerable number of bacteria—such as the diphtheria bacilli, anaerobic sporeforming bacilli, and the common species of cocci—which have been described in the earlier literature as lacking any microscopically demonstrable fat (Lewis, 1941). The preparations revealed in a striking way the abundant lipoid matter in fungi of all kinds.

Intracellular lipid was observed in organisms in primary cultures, as well as in pure stock cultures, and some bacteria and fungi in films made directly from body surfaces or excretions were found to contain typical fatty inclusions.

A high proportion of gram-positive bacteria were discovered to be fat storers. Many familiar varieties of gram-negative bacteria, on the other hand, were shown to be free of stainable lipid when in active growth on common media. A tendency for saprophytic varieties to contain more fat than the parasitic species of the same genera was noted in certain cases, notably in *Mycobacterium* and *Corynebacterium*.

It was found that the appearance of distinct intracellular fat droplets, or other Sudan black B staining matter, in bacteria is not influenced directly by the presence or absence of glycerol, or other fermentable carbohydrate, in the medium. Accumulation of the intracellular lipid is affected, however, in any medium by the rapidity of growth, and if cell division is retarded, the relative amount of demonstrable fat is usually increased. In the case of both the aerobic and anaerobic sporeforming bacilli (genera *Bacillus* and *Clostridium*), the fatty material was observed to be reduced somewhat just before active spore formation began. A considerable amount remained, however, and often sizable fat droplets were seen in the tags of protoplasm around incompletely free spores. If sporulation was for any reason delayed, fatty substances continued to accumulate within the bacilli, and this material persisted indefinitely *in situ*, even after the stainable cytoplasm had disintegrated.

The Sudan black B not only stained all cytoplasmic inclusions of lipoid nature, but also colored parts of the cell structure (apparently the cytoplasmic membrane) in the case of certain bacteria and fungi.

The regularity with which fatty inclusions appeared, and the general pattern exhibited by all the fat-staining material within the cells, were found to be remarkably constant for any one kind of organism. The fat-storing habits of a particular species were not appreciably different in the numerous variant strains observed in this study, whether the variants were naturally encountered or deliberately produced.

Most impressive was the finding that, with only occasional exceptions, the closer the relationship between varieties of bacteria in other respects, the more nearly alike was their content of stainable lipid.

Occurrence of Sudan black B staining material in particular species. The various organisms observed in pure culture may be divided into three groups according to the results of this preliminary survey of their fat-staining propensities (table 1).

In one group (I) stainable intracellular lipid was present regularly in considerable amounts in nearly all the mature cells whenever microscopic examination was made of the growth on the usual culture media. Included here are the larger, common species of *Bacillus;* all representatives of the genera *Clostridium, Corynebacterium,* and *Mycobacterium; Actinomyces* species and the fungi; and some of the more saprophytic gram-positive cocci. Also among the organisms regularly containing conspicuous fatty inclusions are a relatively few species of gram-negative bacteria, including the nitrogen-fixing organisms (*Azotobacter* and *Rhizobium*), and, unexpectedly, such saprophytic species as *Acetobacter aceti, Alkaligenes fecalis,* and *Spirillum rubrum.*

In the case of other bacteria (group II) intracellular fat-staining material was usually present, but the organisms in certain cultures on common media failed to show any fat. These organisms contained relatively small amounts of stainable lipid at most. A clear distinction between these bacteria of group II and those of group I may not be borne out by further investigations. It is convenient, however, to place in this second group, for the present purpose, such organisms as the common, smaller-celled species of the genus *Bacillus*, the human and bovine tubercle bacilli, the familiar varieties of staphylococci, streptococci, and pneumococci, and the gram-negative diplococci, which sometimes grow on the customary media without development of stainable lipid.

No systematic attempt was made to discover the precise circumstances required for the regular formation of intracellular fat by these group II organisms. Incidental observations indicated, however, that at least the majority of them will form characteristic fatty inclusions regularly when a suitable special medium is provided. For example, *Bacillus subtilis* (Marburg) and its close relatives among the aerobic sporeformers rarely show more than traces of fat when cultivated on plain or glucose nutrient agar media. Because of this the writer originally classified these bacilli as "fat-negative" (Burdon, Stokes, and Kimbrough, 1942*a*). But intracellular lipid does appear in characteristic amount when cultures of the same strains are made on potato slants or on glucose starch agar. Similarly, *Lactobacillus acidophilus* apparently stores no fat in milk cultures, but the cells contained moderate amounts when they were grown on tomato juice agar slants.

Finally, the remaining species form a third group made up of bacteria that apparently do not store demonstrable fatty material at all, as a rule, except that one or two tiny droplets are sometimes to be seen in occasional cells (group III). Here are to be found virtually all the chief varieties of gram-negative bacilli,

TABLE 1

An arbitrary grouping of bacteria and fungi according to their content of demonstrable intracellular lipid

(Based on preliminary observations of dried, fixed films stained with Sudan black B and counterstains)

Group I. Fatty Material Regularly Present in Conspicuous Amounts in Nearly all the Mature Cells

Gram-positive bacteria

- *Actinomyces bovis* (1)*
- *Actinomyces* sp. (saprophytic) (3)
- *Bacillus alvei* (1)
- *Bacillus anthracis* (7)
- *Bacillus cereus* (65)
- *Bacillus circulans* (7)
- *Bacillus megatherium* (15)
- *Bacillus mycoides* (10)
- *Clostridium botulinum* (1)
- *Clostridium histolyticum* (2)
- *Clostridium perfringens* (2)
- *Clostridium septicum* (1)
- *Clostridium sporogenes* (4)
- *Clostridium tetani* (3)
- *Corynebacterium diphtheriae* (17)
- *Corynebacterium pseudodiphthericum* (hoffmanni) (3)
- *Corynebacterium xerose* (7)
- *Gaffky tetragena* (3)
- *Mycobacterium "leprae"* (11)
- *Mycobacterium phlei* (3)
- *Mycobacterium smegmatis* (2)
- *Mycobacterium tuberculosis* (avian) (3)
- *Mycobacterium tuberculosis* (cold-blooded type) (2)
- *Mycobacterium* sp. (saprophytic) (8)
- *Sarcina lutea* (3)
- *Staphylococcus citreus* (2)
- *Streptococcus faecalis* (1)

Gram-negative bacteria

- *Acetobacter aceti* (1)
- *Alkaligenes fecalis* (4)
- *Azotobacter beijerinkii* (2)
- *Azotobacter chroococcum* (2)
- *Chromobacterium violaceum* (2)
- *Rhizobium leguminosarum* (2)
- *Spirillum rubrum* (2)

Fungi

- *Aspergillus* sp. (2)
- *Blastomyces dermatitidis* (2)
- *Candida albicans* (2)
- *Coccidioides immitis* (1)
- *Cryptococcus neoformans* (2)
- *Epidermophyton floccosum* (1)
- *Histoplasma capsulatum* (1)
- *Hormodendrum pedrosoi* (1)
- *Microsporum gypseum* (1)
- *Mucor* sp. (2)
- *Penicillium notatum* (1)
- *Penicillium* sp. (3)
- *Phialophora verrucosa* (1)
- *Rhizopus* sp. (2)
- *Saccharomyces cerevisiae* (2)
- *Sporotrichum schencki* (1)
- *Trichophyton mentagrophytes* (1)
- *Rhinosporidium seeberi* (in tissue sections) (1)

Group II. Fatty Material Usually Present, but Sometimes Absent in Cultures on Common Media

Gram-positive bacteria

- *Bacillus mesentericus* (>75)
- *Bacillus subtilis* (Ford) (25)
- *Bacillus subtilis* (Marburg) (>30)
- *Diplococcus pneumoniae* (6)
- *Lactobacillus acidophilus* (2)
- *Mycobacterium tuberculosis* (bovine) (3)
- *Mycobacterium tuberculosis* (human) (8)
- *Staphylococcus albus* (3)
- *Staphylococcus aureus* (3)
- *Streptococcus pyogenes* (3)
- *Streptococcus salivarius* (4)

Gram-negative bacteria

- *Bacillus brevis* (16)
- *Neisseria catarrhalis* (2)
- *Neisseria meningitidis* (2)
- *Neisseria pharyngis* (3)

TABLE 1—*Continued*

GROUP III. FATTY MATERIAL USUALLY ABSENT, OR PRESENT IN TRACES ONLY IN A FEW CELLS

Gram-negative bacteria

- *Aerobacter aerogenes* (2)
- *Brucella abortus* (2)
- *Brucella melitensis* (2)
- *Brucella suis* (2)
- *Eberthella typhosa* (6)
- *Escherichia coli* (2)
- *Escherichia communior* (2)
- *Hemophilus influenzae* (3)
- *Hemophilus pertussis* (2)
- *Klebsiella mutabile* (1)
- *Klebsiella pneumoniae* (2)
- *Listerella monocytogenes* (1)
- *Pasteurella avi* (1)
- *Pasteurella equiseptica* (1)
- *Pasteurella pestis* (2)
- *Pasteurella tularensis* (1)
- *Proteus vulgaris* (OX19) (2)
- *Proteus* sp. (12)
- *Pseudomonas aeruginosa* (3)
- *Pseudomonas fluorescens* (2)
- *Salmonella enteritidis* (2)
- *Salmonella schottmülleri* (2)
- *Salmonella* sp. (4)
- *Serratia marcescens* (3)
- *Shigella dysenteriae* (2)
- *Shigella flexneri* (26)
- *Shigella sonnei* (9)
- *Shigella* sp. (35)
- *Vibrio cholerae* (3)

* The figure in parenthesis indicates the number of separate, pure strains observed by the writer to date.

including all members of the genera *Brucella*, *Hemophilus*, and *Pasteurella*, as well as the several genera of enteric bacilli and the cholera vibrio.

It must be emphasized that this division of bacteria into three groups in relation to intracellular fat is entirely arbitrary and provisional. It is based on films from cultures in routine media only. As the Sudan black B fat stain is eventually utilized by different investigators in connection with intensive studies of various groups of bacteria under different conditions, new information will be forthcoming that may well require revision of these listings.

Characteristic intracellular distribution of the fatty material in important genera and species. The accompanying drawings (figures 1, 2, and 3) give some idea of the usual picture when organisms of various kinds are fat-stained. The sketches can convey only an inadequate conception of the striking appearances actually seen under the microscope, since they lack completeness and full precision in details, and especially since there is an absence of color.

It seems evident that Sudan black B stains more than neutral fat. It not only imparts a dark blue-black color to distinctly outlined intracellular fat droplets, but also gives a more or less intense bluish-gray tint to relatively large, ill-defined areas of cytoplasm in various bacteria and fungi. Moreover, in certain bacteria and most fungi, it stains intensely a thin, irregular, peripheral line, which is clearly part of the cellular structure, and presumably is the cytoplasmic membrane.

Detailed descriptions will not be attempted here, but certain features of general interest will be pointed out.

Bacillus—All members of this genus that have been studied, including virulent strains of *B. anthracis*, store fat (figure 1). Certain small-celled varieties, however, do not do so in every culture, and, moreover, even when stainable lipid is present in these bacilli, it is often difficult to discern. Most commonly the fat occurs in these species at the ends of the rods.

In the larger spore bearers, however, fat forms regularly and early as tiny droplets along the periphery, which soon coalesce into larger drops and move into the middle portions of the cell. The species that are otherwise closely similar (*B. anthracis*, *B. cereus*, and *B. mycoides*) all show large central droplets. *B. megatherium*, on the other hand, presents a distinctly different picture. In this case relatively enormous amounts of lipid are present, even in cultures only a few hours old, and the deeply stained fatty material occurs almost entirely in the form of numerous relatively small droplets, which crowd the cytoplasm but show little tendency to coalesce. The distinctive appearance makes this species easily recognizable.

Clostridium—Considerable intracellular lipid is present in all the anaerobic spore bearers (figure 1). Characteristically, fat droplets occur at the extreme periphery of the counterstained rod, or even appear to be just outside the cells.

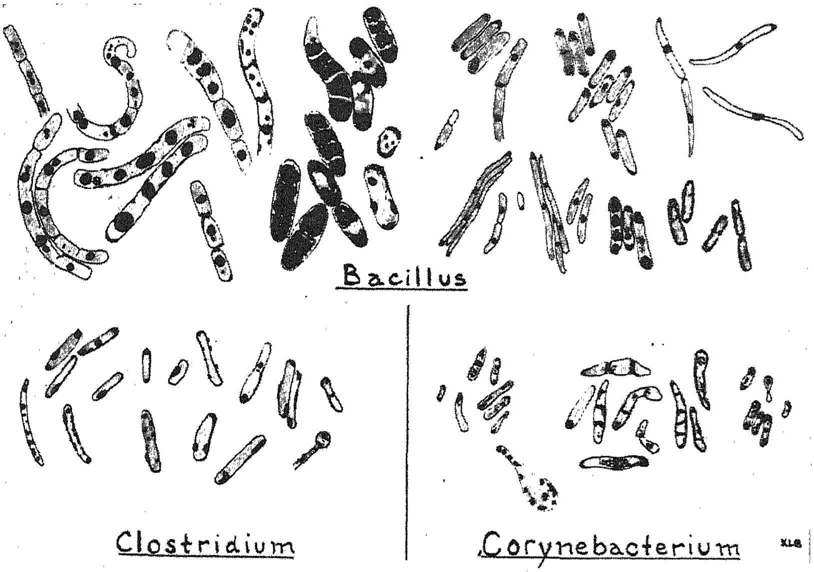

FIG. 1

Corynebacterium—Abundant fatty material is revealed in all the diphtheria bacillus group; it is especially conspicuous in *C. xerose* (figure 1). In this genus the fat stain outlines with bluish black the cell periphery and the cross septa so characteristic of the barred types of these bacilli. Lipid matter also appears as diffuse masses at the ends or edges of the cells.

Mycobacterium—The general picture in the case of all varieties of the acid-fast bacilli is essentially the same There are differences, however, in the number and size of distinctly staining, intracellular fat droplets, and in the regularity with which these appear, among different varieties of these organisms (figure 3). A characteristic feature of the whole group is the tendency of the cells to take a light bluish-gray color throughout. This is in addition to the appearance of distinct, dark-blue droplets of fat within many of the rods.

The human and bovine tubercle bacilli often contain many fat granules, but since we have observed a number of cultures showing only traces of fat, we have listed these organisms in group II (table 1). Unfortunately, no features have been detected which would assist in differentiation of the human from the bovine type. The fat-containing rods have no relation to the "beaded" forms of tubercle bacilli (not infrequently seen after carbol fuchsin staining), whose significance was so long

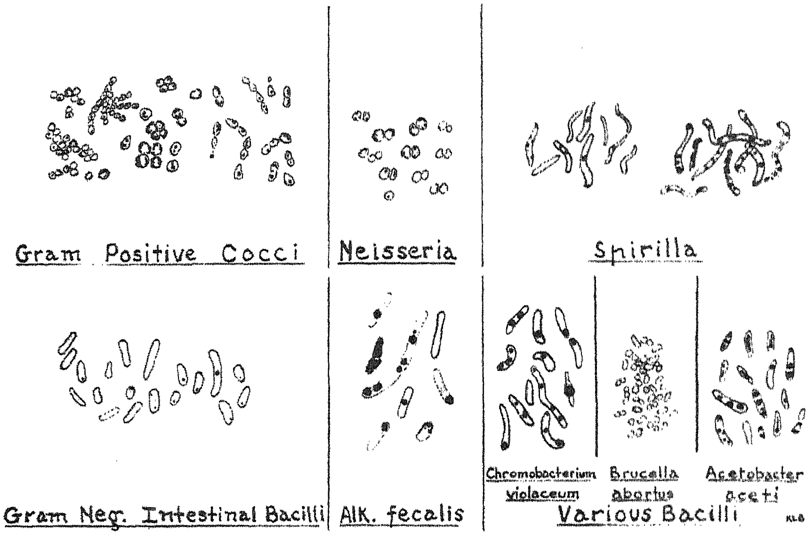

FIG. 2

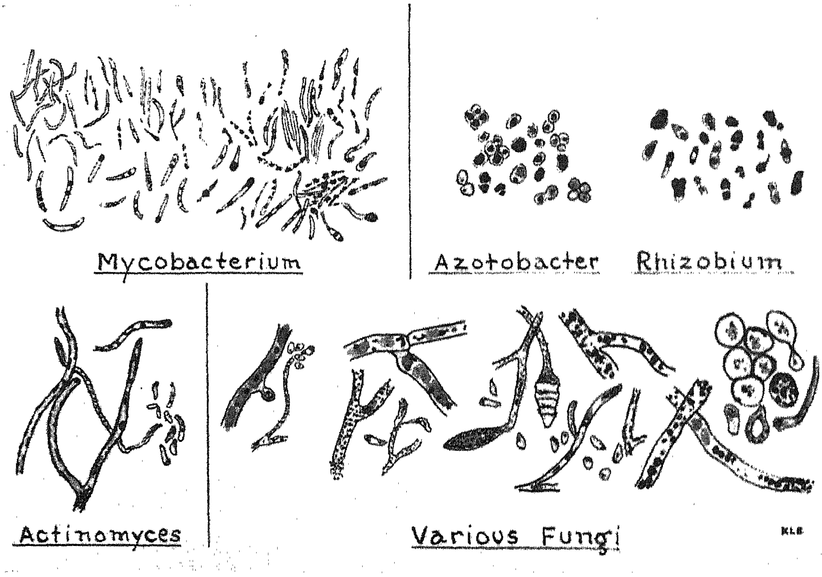

FIG. 3

debated. The "beads" have recently been shown to be artifacts (Porter and Yegian, 1945; Lamanna, 1946).

The most conspicuous fatty inclusions are seen in the frankly saprophytic acid-fast strains, and in cultures labeled *Mycobacterium leprae*. (The bacilli in direct preparations from leprous lesions apparently contain no stainable fat. This disparity in the appearance of the true Hansen's bacilli and the cultured, so-called "leprosy bacilli" when fat-stained is pointed out in a separate communication (Burdon, 1946).

Azotobacter and *Rhizobium*—The extraordinary amount of lipid in the mature cells of the nitrogen-fixing bacteria is revealed in a striking way (figure 3). All stages in the process of accumulating the fat, from the first appearance of almost invisible droplets at the edges of the cells to the final stage in which the entire organism is solidly filled with fat-staining material, may be followed easily.

Cocci, *Actinomyces*, and Fungi—In cocci the Sudan black B staining material most frequently occurs as a dark bluish mantle around the circumference of the cells (figure 2). Some of it also appears as separate, small, internal droplets.

In *Actinomyces* and the filamentous fungi (figure 3) thin strips of lipoid matter or innumerable tiny fat droplets seem to line the hyphae throughout their length. In addition, sizeable round masses and irregular areas within the cytoplasm take the fat stain.

In the yeastlike fungi much fat is also revealed, although it is apparently not as abundant, as a rule, as it is in the mycelium-forming fungi.

Fat in ghost forms; "fatty degeneration." An observation of general interest is the frequent occurrence of fat globules within the "ghost forms" of bacteria, from which stainable cytoplasm is largely or wholly lost. Often these degenerated organisms are packed full of material that takes a dark, blue-black color. These forms appear not only in those species that regularly store lipid, but also in varieties containing no demonstrable fatty inclusions when in their active, growing state. They have been seen, for example, in cultures of *Pasteurella* and *Klebsiella*.

This appears to be a kind of "fatty degeneration." It is probably a phenomenon basically different from the storage of intracellular fat by actively metabolizing cells. Further study is needed before the mechanisms involved can be understood.

Results of staining films from primary cultures and from body secretions. Microscopic examination of colonies of various kinds developing on agar plates exposed to dust showed that the sporulating bacilli and common fungi in such primary mixed cultures contain fat in a characteristic pattern, and that various other fat-positive organisms are likely to be present. Many, but not all, of the bacteria in ordinary hay infusions, and similar mixed growths, were found to contain fat.

Films made from the growth on blood agar plates inoculated with swabbings from the human throat or nose often showed fatty material present in the usual form in various cocci, and in special abundance in diphtheroids. A few of the tubercle bacilli in films of tuberculous sputum were found to contain stainable lipid, and also fat droplets have been seen in the fusiform bacilli in direct preparations from patients with Vincent's angina. On the other hand, no fat has been detected in gonococci in the several films of gonorrheal pus examined. Capsulated anthrax bacilli in direct preparations from the spleen or blood of animals dying of natural or experimental infection were observed to contain only a few very tiny fat droplets along their outer edges. In their very first growth on common media, however, these same organisms always develop characteristic large, centrally located fat granules.

EXTRACTION EXPERIMENTS WITH LIPOID SOLVENTS

As a preliminary step toward some understanding of the nature of the material stained, experiments were carried out to test the loss of Sudan black B staining matter when bacteria are exposed to recognized fat solvents.

Methods. A majority of the extraction tests were carried out by exposing ordinary fixed, but unstained, films to the solvents for varying lengths of time. A number of films were prepared at the same time on separate slides from cultures of various bacteria. These films were fixed by heat as usual, but left unstained, except for one slide of each organism, which was fat-stained to serve as a control. The slides having unstained films were then immersed in the various solvents in Coplin jars. After certain time intervals a slide representing each culture was removed from each of the solvents, stained by the routine procedure, and examined for comparison with the controls. The principal solvents used were glacial acetic acid, 95 per cent ethyl alcohol, acetone, chloroform, and carbon tetrachloride.

A few tests were made by emulsifying the organisms in the solvents directly from cultures. Films were then prepared and stained after varying time intervals.

Results. A summary of the findings in several representative experiments is presented in table 2. It is evident that the Sudan black B staining material was removed in large part from all the organisms by most of the solvents within 72 hours or less. No other change in the microscopic appearance of the bacteria occurred. The rate of extraction varied, however, with different species, as well as with different solvents.

Carbon tetrachloride had scarcely any effect on the fat-staining material in the six varieties of acid-fast bacilli tested, or in *Corynebacterium diphtheriae*, but it removed the lipid matter from five species of aerobic sporeforming bacilli about as readily as the other solvents did. Chloroform also extracted the fatty material from the acid-fast bacilli and from the diphtheria bacillus with relative slowness. The latter organism was the only species not cleared, or nearly cleared, of lipid by acetone within 72 hours. Most rapid extraction was brought about by alcohol, with glacial acetic acid a close second. These two solvents acted only slowly, however, on the closely related, large, chained bacilli *B. anthracis* and *B. cereus*.

The most important influence in determining the extraction rate seemed to be the relative ease with which the solvent was able to enter the bacterial cytoplasm. (It is supposed that this may depend, in part, upon the miscibility of the solvent with water and, in part, upon some peculiarity in the chemical nature of the bacterial cell membranes.) In the case of the slow removal of the lipid from the large bacilli mentioned above, the successive films showed clearly that the delay

TABLE 2

Loss of Sudan black B staining material from bacteria by extraction with fat solvents

ORGANISMS	SOLVENTS														
	Glacial acetic acid			95 % ethyl alcohol			Acetone			Chloroform			Carbon tetrachloride		
	Extraction time (hours)														
	1	24	72	1	24	72	1	24	72	1	24	72	1	24	72
Aerobic sporeforming bacilli															
B. anthracis	−	−	−	−	±	+	−	+	++	+	+++	++++	−	+	++
B. cereus	−	±	+	−	+	++	−	±	+++	−	+	+++	−	+	++
B. megatherium	+	++	+++	+	++	+++	±	+	+++	++	+++	++++	±	++	+++
B. subtilis (Ford)	++++			+++	++++		−	±	+++	±	++++		−	±	+++
B. brevis	+++	++++		+	++++		−	+	+++	−	+	+++	−	+	+++
Acid-fast bacilli															
M. tuberculosis (human, H37)	−	−	++	−	±	++++	−	−	+++	−	−	+	−	−	−
M. tuberculosis (bovine)	−	−	++	−	+	++++	−	+	++++	−	+	++	−	−	−
M. tuberculosis (avian)	−	−	++	±	+	++++	−	+	++++	+	+	++	−	−	±
M. smegmatis	−	−	++	+	++	++++	+	++	++++	−	−	++	−	−	−
M. "leprae"	−	+	+++	−	+	+++	−	−	+++	−	−	++	−	−	−
M. phlei	+	++	+++	+	++	+++	−	±	++	−	±	++	−	−	−
Other bacilli															
A. fecalis	++++			++++			−	+++	++++	+	++++		−	±	+
A. chroococcum	++	++++		−	+++	++++	+	+++	++++	++	++++		−	−	++
C. diphtheriae	+++	++++		−	++++	++++	−	−	±	−	±	+	−	−	−

Key: − = no change; ±, +, ++, +++ = increasing loss of Sudan black B staining material; ++++ = no fat-staining material remaining.

was due chiefly to the slowness with which the solvents were able to penetrate into the cells so as to reach the fatty material. Of course, the relatively large amount of lipid to be dissolved away from these organisms was a factor also.

DISCUSSION

The observations reported here serve chiefly to suggest the potential future usefulness of the staining technique described. More detailed studies with particular groups of organisms will have to be carried out with the aid of the stain before full answers will be obtained to the numerous questions about the intracellular lipid that come to mind.

The origin and fate of fatty inclusions when they occur regularly in actively metabolizing cells, and when they appear in degenerated forms, and the distinction between fat storage as a useful accompaniment of cellular life and the mere accumulation of lipid deposits ("fatty degeneration") are among the matters that should be investigated. From the limited study so far made it seems likely that fat droplets in the cytoplasm originate at the cell periphery, and presumably in some relationship to the cytoplasmic membrane, which itself is apparently colored by Sudan black B in many fungi and in some bacteria. This is in accord with the views of Knaysi (1945, 1946), who has observed the formation of fat droplets from the cytoplasmic membrane in living cells of *Bacillus cereus*. We agree with this investigator that the function (if any) of the fatty inclusions is not clear, and share his opinion that the usual conception of them as droplets of "reserve food material" is inadequate. Although we have noticed indications that some of the intracellular fat is utilized in the late phases of cell growth, most of it (under ordinary cultural conditions, at least) appears to remain unchanged, *in situ*, while the cytoplasm containing it disintegrates.

The precise chemical nature of all the material stained in dried films by Sudan black B is as yet unknown. The dye evidently colors not merely free fat but also lipoid complexes. The extraction experiments demonstrate that recognized lipid solvents will eventually remove all this material from intact bacterial cells, although the rate of extraction differs with the species as well as with the solvent. Study of successive preparations exposed for increasing lengths of time to fat solvents shows that the Sudan black B staining material is truly within the cell, and is gradually removed as the solvent penetrates the cell body. These observations reinforce the conclusion that the substances that color with Sudan black B are not merely surface deposits of stain or other artifacts, but are true cytoplasmic inclusions or integral parts of genuine cell structures. They afford new evidence of the complexity of the bacterial cell (Dubos, 1945).

Of special interest is the finding of definite fat droplets in anaerobic bacteria, both sporeforming and nonsporeforming; this is contrary to the expectations of some authors (Imšenecke, 1945; Meyer, 1912).

An obvious advantage of a fat-staining technique for ordinary dried films is the unlimited opportunity afforded for direct comparisons, at leisure, with the appearance of parallel films of the same organisms treated with other dyes that leave the intracellular lipid unstained. Such comparisons make very clear the important role of fatty inclusions in causing "vacuolation," distortion of the stainable cytoplasm pushed aside by the fat, and "irregular staining" generally. (For a recent study of such staining on the part of the glanders organism, see the article by Worley and Young, 1945.) Lewis (1941) and others have pointed out how the bizarre appearances of fat-containing organisms when colored with methylene blue or other simple stains have led in the past to claims for the presence in various bacteria of "nuclei," "gonidia," and other special structures, and to the idea that such organisms go through complex "life cycles." It is surely no accident that such claims have often been concerned with fat-rich forms (e.g., *Azotobacter*, *Rhizobium*, and *Bacillus megatherium*).

SUMMARY

An improved technique for demonstrating intracellular lipid in microorganisms by staining dried, fixed preparations with Sudan black B and counterstains is described.

The application of this staining method to films of the principal species cultivated on common media revealed that stainable fatty material in the form of cytoplasmic inclusions, or such material associated with structural elements of the cells, is present in all fungi and in the great majority of bacteria, whether these are aerobic or anaerobic, saprophytic or parasitic, pathogenic or nonpathogenic.

A list is given in which the organisms studied are divided arbitrarily into three groups according to their content of demonstrable lipid matter when grown on the media customarily employed for each kind of organism.

With certain notable exceptions (e.g., *Alcaligenes fecalis* and *Azotobacter*), fatty material was found to be more abundant and to occur with greater regularity in gram-positive bacteria than in gram-negative bacteria. An apparent tendency for saprophytic species to contain more fat than parasitic species was noted. Acid-fast bacteria were found to take a bluish-gray color throughout in many instances; the rods were seen to contain distinct, deep-staining fat droplets as well.

Intracellular lipid was discovered in organisms in primary (mixed) cultures, as well as in pure stock cultures, and a number of bacteria and fungi in direct smears from body surfaces and excretions were found to contain fatty material.

Although media of special composition are required for the appearance of stainable lipid in the case of certain bacteria, the formation of fatty inclusions was shown not to be dependent upon the presence of glucose, glycerol, or other fermentable carbohydrate in the medium.

Of greatest interest was the unexpected finding that the relative amount of stainable fatty material and its form and location within the cells of bacteria are remarkably constant for any one kind of organism. Definite differences occur among different kinds, however. Thus, the pattern of intracellular lipid exhibited in the stained films is to a considerable degree characteristic for the bacteria of a particular genus, and in some cases for those of a particular species.

That this is not a mere happenstance is indicated by the further fact that,

throughout the whole phylum, bacteria of species or genera known to be closely interrelated show a marked similarity in their appearance when fat-stained. Indeed, with only occasional exceptions, the closer the relationship between varieties of bacteria in other respects, the more nearly alike is their usual content of stainable lipid. The formation of intracellular fatty material in a particular pattern is evidently a fixed habit in many bacteria, and one that must be associated with something fundamental in the enzymatic or structural make-up of the organisms.

The simplicity of the staining method suggests its routine use in the characterization of microorganisms. Its value as an aid in the differentiation of species and genera will be more fully established by further trials. Its possible usefulness in practical diagnostic work is largely unexplored. The stain should prove especially helpful in future investigations of bacterial cytology and metabolism.

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DISPARITY IN APPEARANCE OF TRUE HANSEN'S BACILLI AND CULTURED "LEPROSY BACILLI" WHEN STAINED FOR FAT

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By application of the writer's improved fat-staining procedure for dried preparations (Burdon, 1946) it was found that the principal varieties of the acid-fast bacilli in culture show an essentially similar picture with respect to their stainable intracellular lipid. Characteristic of the whole group is the tendency of the cells to stain *throughout* with the Sudan black B; in addition, distinct deeply colored fat droplets may be present within many of the rods. Some differences were noted, however, in the amount of fatty material usually present, and in the regularity with which it occurred, in different varieties of these organisms.

When preparations were made from parallel cultures on coagulated egg media, the 8 strains of human and the 3 strains of bovine tubercle bacilli examined usually contained a moderate number of fat droplets, but there were always to be seen numerous individual rods without stained lipid, and in some cultures none of the organisms were found to contain any material that colored with Sudan black B. Three strains of avian tubercle bacilli, however, regularly showed fatty cell inclusions in almost every mature cell. Stainable intracellular lipid was also found constantly, and more abundantly, in 2 strains of tubercle bacilli of cold-blooded animals (Friedmann's turtle bacillus and *Mycobacterium marinum*) and in 8 cultures of frankly saprophytic acid-fast organisms.[1] Finally, in all the 11 cultures of "*Mycobacterium leprae*" studied[2] the stain revealed an especially conspicuous content of fatty material.

The constancy and prominence with which stainable lipid occurs in these cultured "leprosy bacilli" was repeatedly confirmed, and their marked similarity in this respect to the tubercle bacilli of the "cold-blooded type," and to the frankly saprophytic acid-fast organisms, was made clear by numerous comparative tests. In the light of our finding that closely related bacteria tend to have the same content of fat-staining material (*loc. cit.*) this similarity is suggestive of a fundamental biological relationship between these organisms.

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[1] The saprophytes included cultures labeled as follows: (1) Hog skin bacillus 5138, Dr. Buckley, B.A.I. 1926, ATTC 4268, Group IIIb; (2) Zeissig's acid-fast bacillus, Cornell, 1927, Group IIIa; (3) *Mycobacterium phlei*, Lister Inst. 59, ATCC 354; (4) Butter bacillus of Rabinowitsch, Lister Inst. 524, ATCC 356, Group Ia; (5) *Mycobacterium pseudoperlsucht*, Lister Inst. 2070, Group Ia; (6) Bayne-Jones acid-fast bacillus, Group IIa (1); (7) *Mycobacterium phlei* (timothy), 223 (Pasteur Inst.); (8) *Mycobacterium phlei*, ATCC 355, E. G. Hastings' 74B. Cultures (1) through (6) were obtained from the N. Y. State Veterinary College, through the kindness of Dr. W. A. Hagan.

[2] Eight cultures of "leprosy bacilli," received from the National Leprosarium, Carville, La., were marked as follows: No. 111, isolated by Levy (Chrome) (Duval 114); No. 116, isolated by Neaham (64) (Ann Arbor 68a); No. 121, isolated by Clegg (Ann Arbor 0580); No. 122, isolated by Kral (Ann Arbor 0614); No. 132, isolated by Souza-Aranjo (Souza-Aranjo I); Elly strain; Barry strain; Phipps Inst. strain. Another culture obtained from N. Y. State Veterinary College was labeled Clegg I, Lister Inst. 512, 1926, Group Ib. Two additional cultures, from our stock culture collection, were marked Brinkerhoff 1, Duval 107, from Duval 105; and Brinkerhoff 2, Duval 108.

In these circumstances it seemed of special interest to apply the stain to direct films from leprous lesions, and thus to extend observations to the true Hansen's bacilli. Through the kindness of Dr. G. H. Faget, Medical Director, U. S. Marine Hospital (National Leprosarium), Carville, Louisiana, a series of unstained preparations made directly from bacteriologically positive lesions was secured. These preparations were subjected to the routine fat stain, and to various modifications of the usual technique, in order to test critically for the presence of stainable lipid in the true leprosy bacilli. The procedure finally adopted was as follows: (1) the heat-fixed films were stained with dilute carbol fuchsin only and examined microscopically to locate the leprosy bacilli (recognizable by characteristic clumps and globi); (2) several of these groups of bacill were marked by making a ring around them on the slide with a Zeiss object-marker; (3) after removing the immersion oil by a brief dipping in xylol, Sudan black B solution was applied for 15 minutes or longer; then the smear was cleared with xylol as usual, and sometimes it was also counterstained with dilute carbol fuchsin, sometimes not; (4) the ringed areas were relocated and the organisms were carefully examined for any evidences of stained intracellular lipid; (5) the slides were again cleared of oil and subjected to the usual Ziehl-Neelsen acid-fast stain; and, finally, (6) the organisms in the ringed areas were located once again in order to confirm their identity as leprosy bacilli by their acid-fast staining. Satisfactory controlled observations of this sort were possible on a total of 15 preparations, representing 8 separate leprous patients.

The results were entirely consistent in all preparations—that is, no intracellular stainable fatty material was observed in any of the Hansen's bacilli. This finding is given somewhat added weight by our further observation that human tubercle bacilli *do* contain matter which stains with Sudan black B in direct preparations from tuberculous tissues, as well as in most cultures. The apparent total lack of stainable lipid in the true causative bacilli of leprosy is, at least, in striking contrast to the abundance of this material in the acid-fast bacilli isolated from leprous lesions and now maintained in the laboratory cultures described above. The full significance of this disparity is debatable at this time, but it would seem justifiable to count it as adding a further bit of evidence in support of the already widely held opinion that the organisms in these cultures are not identical with the true causative agent of leprosy. Their conspicuous content of stainable lipid strongly suggests a close relationship to saprophytic acid-fast bacilli.

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COMPARATIVE ACTION OF AN EXTRACT OF BRAIN TISSUE AND PENICILLIN ON STAPHYLOCOCCUS AUREUS INFECTIONS

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Carefully controlled clinical studies have been made (Chain *et al.*, 1940; Lyons, 1943; Robinson, 1943) of the value of penicillin and the sulfonamides in various infections, and the production and use of these antibiotics has attracted considerable attention to other potential sources of antibiotic agents (Waksman, 1945).

For a number of years our laboratories have investigated the effects on bacterial growth of certain substances obtained from plant and animal sources. Some extracts have been found to stimulate, other to inhibit, growth (Sperti *et al.*, 1937; Fardon and Sullivan, 1938–1939; Cook *et al.*, 1941; Schroeder and Hollencamp, 1941). A substance obtained from both human and beef spleen has a germicidal action against *Streptococcus pyogenes, in vitro* (Nutini and Kreke, 1942). A factor extracted in a similar manner from beef spleen, heart, kidney, and brain produced a conversion *in vitro* of the original yellow S form of *Staphylococcus aureus* to a white R configuration, the latter showing altered biochemical features (Nutini and Lynch, 1945). The converted organism retained its altered morphologic and biochemical characteristics *in vivo*, producing no evidence of virulence when injected into mice (Nutini and Lynch, 1946). The extracts of beef spleen, heart, kidney, and brain were effective against *Staphylococcus aureus* infections induced subcutaneously, intravenously, and intraperitoneally (Nutini and Lynch, 1946). The mortality in experimental animals receiving *S. aureus* and the brain extract subcutaneously was 0.9 per cent in the prophylactic series of 223 animals and 3 per cent in the therapeutic series of 116 animals, mortality in the control series being 75 and 87 per cent, respectively. The healing time for the small, dry, atypical lesions in the prophylactic series averaged 7 days and ranged from 3 to 14 days; for the typical suppurative lesions the healing time in the control series was 18 days (range 9 to 30). The brain extract was effective orally as well as subcutaneously against the infections. It is relatively nontoxic, as much as 400 mg, or 2 per cent of the body weight of the experimental animal, having been given daily for 10 days without ill effect in mice.

The present paper is a report of the comparative value of brain extract and penicillin as therapeutic and prophylactic agents against *S. aureus in vivo.*

The virulent strain of *S. aureus* was obtained from ATCC 6636, and 48-hour broth cultures were used for subcutaneous inoculations of 0.5 ml (1.5 LD_{50}) into the ventral abdominal region of the test animals, which were brown black mice of the BBC strain, 3 to 6 months old.

The brain extract, prepared as previously described (Nutini and Lynch, 1946), was administered subcutaneously in the ventral abdominal region in doses of 50 mg daily. In the prophylactic experiments the first injection was given 2 hours before inoculation with the *S. aureus*. In the therapeutic experiments treatment was begun on the third day following inoculation with the infecting organism, at which time there were typical suppurating lesions. The control animals received 0.25 ml of saline daily.

The penicillin was the commercial sodium salt manufactured in the Cheplin Laboratories. The dosage was 750, 1,000, and 2,000 Oxford units per day, given subcutaneously in divided doses at 6-hour intervals in the ventral abdominal region. The procedure for inoculating the mice with the infecting organism was the same as that for the animals treated with brain extract. When the experiments were repeated, the injections of penicillin were given at 12-hour intervals. No difference was observed in the response from that of animals treated at shorter intervals. The dosage levels were 5 to 10 times greater than the maximal amounts used by Robinson (1943) in the treatment of staphylococcic infections in mice.

TABLE 1

Comparison of the prophylactic action of brain extract and penicillin on subcutaneous Staphylococcus aureus infections in mice

GROUP	NO. OF ANIMALS	FREQUENCY AND TYPE OF ABSCESS	MORTALITY	TIME OF HEALING FOR SURVIVORS, AVG RANGE
		per cent	*per cent*	*days*
Control	20	100 severe	90	23 (23)
Brain extract, 50 mg/day	20	80 needle point 20 moderate	0	7 (4–9)
Penicillin, 750 u/day	10	20 moderate 80 severe	70	13 (10–17)
Penicillin, 1,000 u/day	20	100 moderate	25	11 (8–16)
Penicillin, 2,000 u/day	10	20 none 80 moderate	10	9 (6–14)

Staphylococcus culture, ATCC 6636; dose used, 1.5 LD_{50}.

In the first of the prophylactic series of experiments, 40 mice were used, 10 serving as infected control animals, 10 receiving 50 mg of brain extract per day, and 20, in groups of 10, receiving penicillin in daily doses of 750 and 1,000 Oxford units, respectively. Daily treatments were continued until the lesions were healed as judged by the scabs dropping off and leaving the smooth new skin beneath. When the experiment was repeated, a dose of 2,000 Oxford units per day of penicillin was substituted for the 750-Oxford-unit dose per day.

In the therapeutic experiments subcutaneous inoculations were made with 0.5 ml(1.5 LD_{50}) of the virulent 48-hour broth culture of *S. aureus*, and 3 days later, after the development of typical lesions, the animals were divided into 4 groups, each containing mice with infections of similar severity. One group of 10 served as infected, untreated control animals, and the other groups received, respectively, 50 mg of brain extract per day, and 1,000 and 2,000 Oxford units of the sodium salt of penicillin per day in divided doses at 6-hour intervals until healing was complete. In the second experiment the doses of penicillin used were 750 and 1,000 Oxford units per day.

RESULTS

Prophylactic experiments (*table 1*). All of the control mice developed suppurating lesions that were typical in size and appearance for staphylococcic infections. In those animals receiving the brain extract 2 hours before inoculation with the *S. aureus*, needle-point lesions at the site of the injection developed in 80 per cent of the animals; in 20 per cent there were atypical, small, dry, nonsuppurating lesions, which healed within 4 to 9 days. In the animals treated with penicillin, 100 per cent of those that received 750 and 1,000 Oxford units per day and 80 per cent of those that received 2,000 Oxford units per day developed suppurating lesions that were smaller than those in the control animals but were larger than the nonsuppurating lesion characteristic of the animals receiving the brain extract. As shown in table 1, the average healing time for the groups of animals receiving penicillin was 13, 11, and 9 days, the interval being the shortest with the highest dosage of penicillin.

TABLE 2

Comparison of the therapeutic action of brain extract and penicillin on subcutaneous Staphylococcus aureus infections in mice

GROUP	NUMBER OF ANIMALS	MORTALITY	AVERAGE TIME OF HEALING FOR SURVIVORS
		per cent	*days*
Control	20	95	27 (27)
Brain extract	20	0	7 (4–11)
Penicillin, 750 u/day	10	80	23 (21–26)
Penicillin, 1,000 u/day	20	45	17 (11–19)
Penicillin, 2,000 u/day	10	10	19 (18–22)

Staphylococcus culture, ATCC 6636; dose used, 1.5 LD_{50}.

Therapeutic experiments (*table 2*). In the animals treated with brain extract, the suppurating lesions typical of staphylococcic infections in mice apparently began to dry up as early as the second day following initiation of treatment, the lesions developing a dry, hemorrhagic appearance with the crust dropping off on the sixth to the ninth day. In the groups of animals receiving penicillin, suppuration continued for 3 to 9 days, with the first signs of healing appearing on the seventh day. None of the animals receiving brain extract died. In the groups receiving penicillin the mortality was 80, 45, and 10 per cent, the lowest mortality occurring in the animals receiving the highest dosage. Healing required, on an average, 23, 17, and 19 days in the survivors of the penicillin-treated animals.

The results of experiments with penicillin-resistant strains of *S. aureus* and on the possible development of resistance to brain extract by these organisms are to be published in a separate communication.

CONCLUSIONS

It is apparent from the evidence presented that there is in brain tissue a factor that functions effectively against *Staphylococcus aureus in vivo.* Whether the extract is used as a prophylactic or therapeutic measure, it is superior to penicillin in the dosages used for *Staphylococcus aureus* infections in these experiments.

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THE SO-CALLED PSEUDOMONAS VENDRELLI

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In 1938 pure cultures of a *Pseudomonas* were recovered in large numbers from Mr. Fernando Vendrell's shallow well at Canas, near Ponce, Puerto Rico. No other bacteria were found in the water. Since it was known that *Pseudomonas aeruginosa* sometimes caused dysentery or typhoidlike infections, the water was reported as being of undesirable sanitary quality. A similar case has been reported (Rochaix and Vieux, 1937) in which *P. aeruginosa* apparently exerted such a strong antagonistic action upon other bacteria in water that counts for coliform organisms failed to give an index of the poor sanitary quality of the water.

Although showing some resemblance to *P. aeruginosa*, the organism showed much stronger pigmentation than ordinary laboratory strains of that organism. It also appeared to differ in certain cultural characteristics from the description given in Bergey's *Manual of Determinative Bacteriology*, fourth edition, 1934. More detailed reference facilities were not readily available. Therefore cultures were sent to other laboratories (where it was hoped that a definite identification might be made) under the tentative name of *Pseudomonas* (*Bacillus*) *vendrelli*. However, the recipients of the cultures accepted this unfortunate *nomen nudum* as valid, and the organism was so mentioned in the printed literature (Farrell and Wolff, 1941; Lockwood *et al.*, 1941, 1942; Lasseur *et al.*, 1945).

In 1941 the organism was found to produce considerably larger amounts of proteolytic enzymes than five other *Pseudomonas* strains and species, so that some work was done in this laboratory to develop its use in the production of a bating agent for leather. This work was later discontinued for economic reasons.

Still later, in 1942, other workers in this laboratory found it to have strong antibiotic properties, particularly against gram-positive bacteria. The yields of the active material were variable, but were generally at a maximum on media containing Armour's peptone, lot 100891, manufactured before World War II. Other lots of Armour's peptone, and most other brands of peptone, generally yielded much less antibiotic.

Upon a recheck of cultural characteristics and correspondence with others who had handled the organism, it ultimately became apparent that it was a strain of *P. aeruginosa*. It was quite unstable in its cultural characteristics. However, it usually produced exceptionally large amounts of fluorescent yellow-green pigment in young cultures. Pyocyanin appeared later, and old cultures contained large amounts of the red pigment pyorubrin, described by Meader *et al.* (1925). The antibiotic proved to be pyocyanase, a substance that is of little practical utility on account of its hemolytic properties. The work of Gaby

(1946) and others also indicates that many strains of this species are unstable and frequently puzzling in their cultural and other characteristics.

The history of this strain illustrates the confusion which may arise when a supposedly new species is isolated, given a provisional specific name, and sent to various laboratories. A much better practice in such cases is to designate the genus (if this is known) and to append a serial number, omitting any specific name in order to avoid the misleading implication that the organism is actually a new species described in some publication.

It is also evident that, in certain cases, a taxonomic study of an unidentified organism may be helpful in giving a clue to the probable identity of any antibiotic substance produced. If our organism had been identified initially as a strain of *P. aeruginosa*, the possibility that the antibiotic was pyocyanase would have been apparent immediately, and some unnecessary work could have been avoided. However, since different strains of the same species may produce different antibiotics, and totally different species sometimes produce the same antibiotic, the value of taxonomic work should not be overstressed. It is always desirable, however, to identify an unknown organism definitely before publishing anything about it. The journal literature contains far too many papers that are of very little value since the organisms in question are either not identified or are described only very inadequately.

The so-called *Pseudomonas vendrelli* should be redesignated as *Pseudomonas aeruginosa*, Vendrell strain. Its unusually strong production of proteolytic enzymes and of fluorescent pigment, as well as its apparent power (at least as found in nature) to inhibit other bacteria, give it some theoretical interest. It is available from the American Type Culture Collection, Washington, D. C., as their culture no. 7700.

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FACTORS INFLUENCING THE MORPHOLOGY OF BLASTOMYCES DERMATITIDIS

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Blastomyces dermatitidis possesses two morphological forms, a yeastlike form in tissue and a mycelial form on Saboraud's medium at room temperature. A yeastlike form similar to the form appearing in tissue may be obtained if the organism is grown on blood agar at 37 C.

An extensive literature has developed around blastomycosis as a clinical entity, but only a small proportion of the papers have been concerned with the biological nature of the etiological agent, *Blastomyces dermatitidis*. Ricketts (1901) described the two morphological forms. Hamburger (1907) concluded that temperature was the most important factor influencing morphology, and that room temperature favored mycelial formation, but incubator temperatures favored the yeastlike form. He did not determine the effect of intermediate temperature or of hydrogen ion concentration. Michelson (1928) studied the effect of higher temperatures and noted that temperatures of 37, 41, and 45 C favored the growth of the yeastlike form. Moore (1933) studied the organism for the purpose of determining its position in botanical classification. He studied its growth in media of different hydrogen ion concentrations, but each hydrogen ion concentration was represented by a different medium.

The purpose of the present study was to determine the effect of various controlled changes in the environment, such as nutrition, hydrogen ion concentration, temperature, and type of inoculum on the two morphological forms of *Blastomyces dermatitidis*.

METHODS

Media. Solid media were used for all the studies, since preliminary work indicated that the organism grew best under purely aerobic conditions, and that the yeast form grew very poorly in liquid media.

Preliminary nutritional studies showed that the organism grew as well as or better on a peptone glucose medium than it did on extract blood agar. The organism grew equally well on a medium consisting of salts, glucose, and vitamin-free casein hydrolyzate, indicating that it does not require the addition of any of the ordinary accessory factors. Growth also occurred on a medium consisting of salts, glucose, and ammonium sulfate, but never equalled that occurring on the more complete peptone glucose medium. This indicates the stimulatory action of amino acids.

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Two different media were employed: (1) a complete medium represented by the peptone glucose medium; and (2) a deficient medium represented by the ammonium sulfate glucose medium.

Peptone glucose medium		*Ammonium sulfate glucose medium*	
Peptone	0.5 g	$(NH_4)_2SO_4$	0.5 g
Glucose	1.0 g	Glucose	1.0 g
K_2HPO_4	0.5 g	K_2HPO_4	0.5 g
KH_2PO_4	0.5 g	KH_2PO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g	$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.01 g	NaCl	0.01 g
$FeSO_4 \cdot 7H_2O$	0.01 g	$FeSO_4 \cdot 7H_2O$	0.01 g
$MnSO_4 \cdot 2H_2O$	0.0065 g	$MnSO_4 \cdot 2H_2O$	0.0065 g
Agar	1.5 g	Agar	1.5 g
H_2O	100 ml	H_2O	100 ml

Portions of each of these media were adjusted to hydrogen ion concentrations of pH 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, and 9.5 and dispensed into tubes. All tubes were autoclaved at 15 pounds' pressure for 15 to 20 minutes and slanted.

Inoculum. Two types of inocula were used, a suspension of the yeastlike cells and a spore suspension. A 3- to 5-day blood agar culture of the yeast phase was suspended in sterile physiological saline and washed. The suspension of the washed cells was brought to a standard turbidity by means of a lumetron model 400 G photoelectric colorimeter (60 per cent transmission, blue filter wave length 420 A°). One drop of the standard suspension was delivered from a Wright pipette into each tube and then spread over the surface of the agar slant with a sterile wire loop.

The spore suspension was prepared in sterile distilled water by gently scraping the surface of a 2- to 4-week-old culture grown at room temperature. The suspension consisted primarily of blastospores with a minimum amount of mycelial fragments. A drop of this suspension was delivered into each tube and spread with a wire loop. All tubes were inoculated in duplicate.

The inoculated tubes were incubated at room temperature and in water baths at 31, 33, 35, and 37 C. The water baths were capable of maintaining a constant temperature within 0.5 C. Observations were made at 3, 5, 8, 11, and 15 days. The amount of growth was estimated visually and the type of growth was also determined by microscopic examination. No attempt was made to follow the pH during the course of growth, since the medium was well buffered and any changes that might have occurred would have been common to all the tubes. The validity of the observations was confirmed by means of repeat experiments.

Organism. The strain of *Blastomyces dermatitidis* used for the study was originally from the culture collection at Duke University. A duplicate series of experiments with two other strains of *Blastomyces dermatitidis*[2] confirmed the results obtained with the original strain.

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[2] The other strains of *Blastomyces dermatitidis* used were as follows: a strain originally obtained from the culture collection of the late Dr. A. T. Henrici and a recently isolated strain obtained from a patient at the Research and Educational Hospital of the University of Illinois.

RESULTS

Peptone glucose medium: Yeast inoculum. Growth on this medium was maximal. Using the yeast cell inoculum, growth was visible in 2 to 3 days, regardless of the resulting morphology. From room temperature through 33 C the organism grew as the mycelial form. At 35 and 37 C the organism grew as the yeastlike form. This occurred irrespective of the hydrogen ion concentration.

Each form of the organism possessed an optimum temperature at which growth proceeded at the most rapid rate. For the mycelial form this temperature was 31 C, but for the yeastlike form it was 35 C.

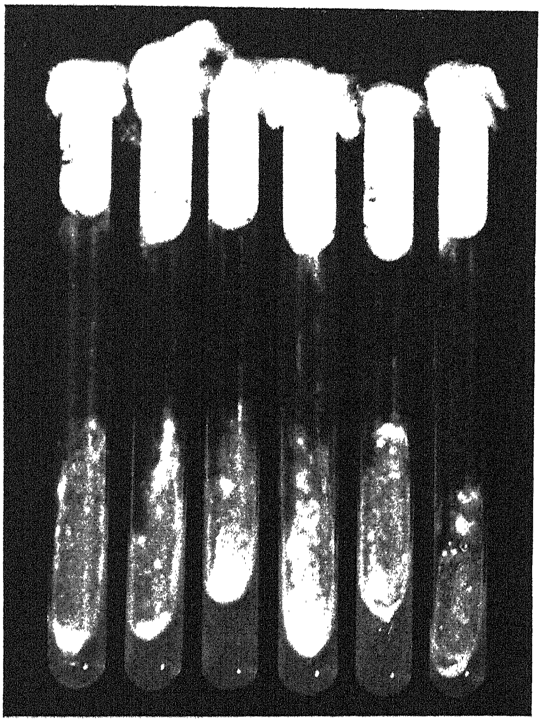

FIG. 1. SHOWING THE EFFECT OF TEMPERATURE ON THE GROWTH OF BLASTOMYCES DERMATITIDIS ON A PEPTONE GLUCOSE MEDIUM AFTER FIVE DAYS

From left to right, the tubes are arranged as follows: 2 tubes incubated at room temperature, 2 tubes incubated at 31 C, and 2 tubes incubated at 33 C.

At its optimum temperature (31 C) the mycelial form grew at all hydrogen ion concentrations used. Its growth was retarded at pH 3.5, 8.5, and 9.5. The growth at pH 8.5 and 9.5 eventually reached a maximum very close to that attained at the more optimum hydrogen ion concentrations, pH 4.5 to 7.5. The formation of aerial mycelia was restricted at pH 4.5 and below. At room temperature, the organism grew well at all hydrogen ion concentrations with the exception of pH 3.5. Growth was retarded at pH 8.5 and 9.5, but the amount of growth eventually equalled that attained at the more optimum hydrogen ion concentrations. The formation of aerial mycelia was somewhat restricted at the greater hydrogen ion concentrations. Although growth proceeded at a slower rate, the maximum growth attained eventually equalled that occurring at 31 C. Growth at 33 C was not so good as at the lower temperatures. The amount of growth was restricted and the rate of growth was retarded at pH 4.5, 5.5, 8.5, and 9.5. The best growth occurred at pH 5.6 to 7.5. The formation of aerial mycelia was restricted at all hydrogen ion concentrations. This effect, however, was particularly marked at the greater hydrogen ion concentrations. There was never any growth at pH 3.5.

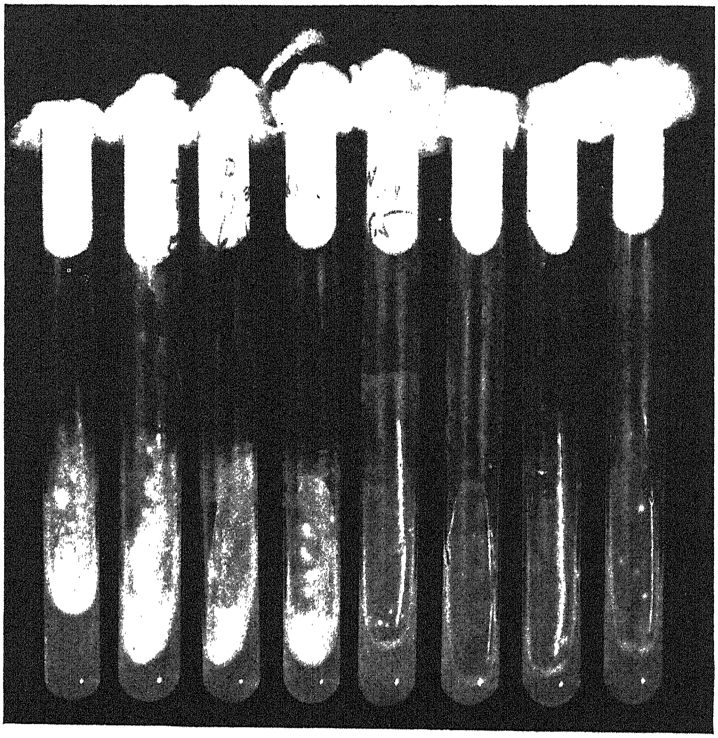

FIG. 2. SHOWING THE EFFECT OF NUTRITION ON THE GROWTH OF BLASTOMYCES DERMATITIDIS INCUBATED AT 31 C FOR 5 DAYS

The tubes are arranged in groups of 4 for each medium and from left to right are as follows: the peptone glucose medium and the ammonium sulfate glucose medium.

At its optimum temperature (35 C) the yeastlike form grew at all hydrogen ion concentrations except pH 3.5. The optimum range of hydrogen ion concentrations for growth was pH 5.5 to 8.5. Growth was greatly restricted at pH 4.5 and retarded at pH 9.5. At 37 C no growth occurred at pH 4.5 or below. The growth at the other hydrogen ion concentrations was slower in starting than at the corresponding hydrogen ion concentrations at 35 C, but the maximum growth attained was the same.

Spore inoculum. The results obtained when the spore suspension was used to inoculate the medium were essentially the same as those observed with the the yeast phase inoculum. The temperature at which the transition between the yeast and mycelial phases occured was the same, i.e., between 33 and 35 C. The optimum temperatures for the two forms were also the same, 31 C for the mycelial form and 35 C for the yeastlike form. It takes longer for growth to get started when this type of inoculum is used. Whereas growth was apparent at 2 to 3 days when yeast cells were used as the inoculum, it took 3 to 5 days to get comparable growth with the spore inoculum. This was primarily due to the length of time necessary for germination of the spore. Therefore, at any one time when this inoculum was used, the amount of growth was always less than when the yeast phase was used. The conditions for germination are apparently more rigid than for cell multiplication, as evidenced by the fact that the pH limits for growth were more restricted. Growth at pH 4.5 was delayed for more than 5 days and then never approached the growth observed at the lower hydrogen ion concentration. Growth was also restricted at pH 8.5 and 9.5. No growth occurred at pH 4.5 or below at 37 C.

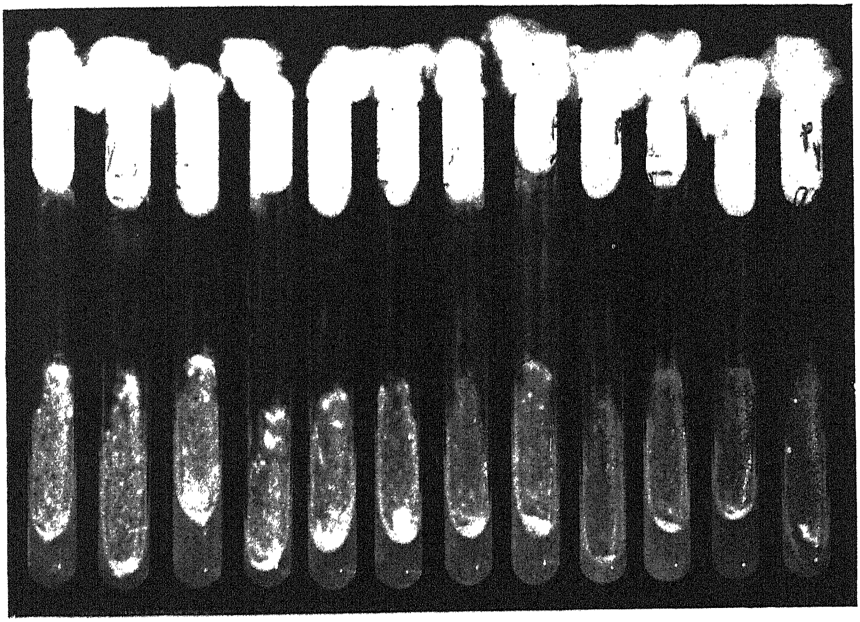

Fig. 3. Showing the Effect of Hydrogen Ion Concentration on the Growth of Blastomyces dermatitidis Incubated at 33 C on a Peptone Glucose Medium for 5 Days

The tubes are arranged in pairs for each hydrogen ion concentration, as follows, from left to right: pH 4.5, 5.5, 6.5, 7.5, 8.5, and 9.5.

In general, it would seem as if the range of hydrogen ion concentrations at which good growth will occur becomes more restricted as the temperature increases.

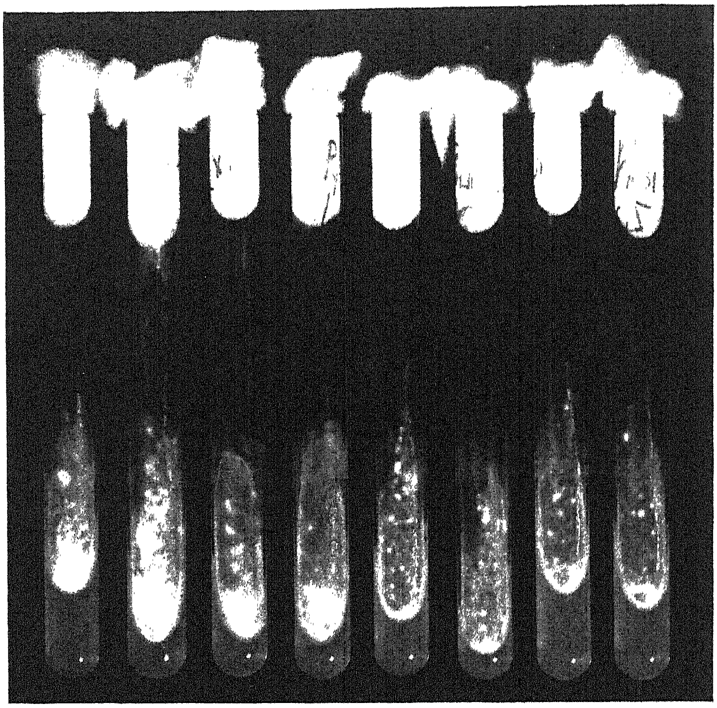

Fig. 4. Showing the Effect of the Type of Inoculum on the Growth of Blastomyces dermatitidis Incubated at 31 C on a Peptone Glucose Medium for 5 Days

The tubes are arranged in groups of 4 for each type of inoculum, as follows from left to right: yeast phase cell suspension and spore suspension.

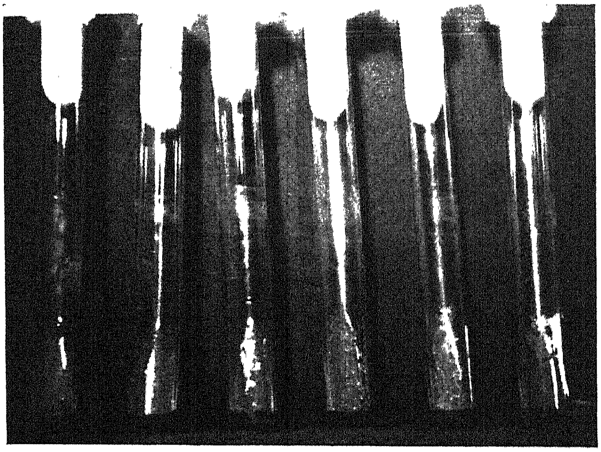

Fig. 5. Showing the Effect of Hydrogen Ion Concentration on the Growth of Blastomyces dermatitidis Incubated at 37 C on the Ammonium Sulfate Glucose medium for 5 days

From left to right the tubes are as follows: pH 4.5, 5.5, 6.5, 7.5, 8.5, and 9.5.

Ammonium sulfate glucose medium. Growth on this deficient medium assumes a different pattern from that occurring on the complete peptone glucose medium. The amount of growth never equalled that on the peptone medium. The effect of hydrogen ion concentration is different. On the peptone glucose medium there occurred an optimum hydrogen ion concentration somewhere between pH 5.5 and 7.5 to 8.5 with growth falling off on either side. On this medium, regardless of the temperature and therefore of the morphological form assumed by the organism, the quantity of growth increased as the hydrogen ion concentration decreased. When the pH reached 9.5, there occurred a slight decrease in the quantity of growth. The effect of temperature was also different. Although the transition between the mycelial and yeastlike forms took place at the same temperatures, that is between 33 and 35 C, the optimum temperature for each form was different. In general, the higher the temperature, the greater the amount of growth. This effect was not so apparent with the mycelial phase (i.e., from 31 to 33 C) as it was with the yeast phase (35 to 37 C). The amount of growth occurring at 37 C was very much greater than that occurring at 35 C. These effects were observed with both the yeast and spore inocula.

There was a tremendous difference in the amount of growth occurring when the different inocula were used. Although, as stated above, growth on this medium never approached that occurring on the peptone glucose medium, the tubes inoculated with the yeastlike phase exhibited a great deal more growth than those inoculated with the spore suspension. When the spore suspension was used for inoculation, no growth occurred at room temperature and practically none occurred at 31 and 33 C. Practically no growth occurred even at the lower hydrogen ion concentrations. This inability of the spore suspension to initiate growth to any extent on this deficient medium again demonstrates the more rigorous conditions necessary for spore germination.

ACKNOWLEDGMENT

The authors are indebted to Dr. Esther Meyer for supplying the cultures of *Blastomyces dermatitidis* used in this study.

CONCLUSIONS

The most important factor affecting the change in morphology of *Blastomyces dermatitidis* is temperature.

On a complete medium, each morphological form possesses an optimum temperature for growth, 31 C for the mycelial form and 35 C for the yeastlike form.

On the complete medium, both forms tend to grow at all hydrogen ion concentrations used, except pH 3.5. At 37 C, however, there is no growth at pH 4.5 and below.

On the deficient medium, the opimum temperature and the effect of hydrogen ion concentration are different. In general, the higher the temperature, the greater the amount of growth exhibited by each form. Therefore, for the mycelial form the optimum temperature is 33 C, and for the yeastlike form the optimum temperature is 37 C. As the hydrogen ion concentration decreases, the amount and rate of growth increases to a maximum at pH 8.5 for both forms. The amount of growth is somewhat decreased at pH 9.5.

The tendency to form aerial mycelia decreases as the hydrogen ion concentration and the temperature increase.

Blastomyces dermatitidis does not require any of the commonly known accessory factors for growth. Amino acids have a stimulatory role.

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STAGES IN THE LIFE HISTORY OF PHYTOMONAS TUMEFACIENS

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Phytomonas tumefaciens, the root nodule organisms, and other related bacterial species may produce, in the course of their life histories, peculiar, star-shaped aggregates of cells. Beijerinck (1890) and Beijerinck and van Delden (1902) were apparently the first to describe this phenomenon. Working with the root nodule bacteria and *Alcaligenes radiobacter* these investigators interpreted this phenomenon as being the result of a peculiar type of branching or division of a single bacterium. At the time when Löhnis (1921) was formulating his life cycle theory, he also observed these star-shaped bodies and concluded that they represented evidence of conjunction in the bacteria.

In his first report on the subject Lieske (1926) did not attempt to interpret the phenomenon but later (1928) suggested that, superfically at least, it had the appearance of a sexual stage in the life history of the bacterium studied. Stapp and Bortels (1931) presented a detailed account of the different phases of the life cycle of *Phytomonas tumefaciens*. These investigators devised a simple medium which permitted the initiation and development of large numbers of bacterial stars and thus greatly facilitated their study. The medium consisted of dilute carrot juice and 0.01 per cent each of $FeSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot 7H_2O$. Recently Stapp (1942) has studied, with the aid of the Feulgen stain, the nuclear behavior within the aggregate. He has suggested that fusion of the nuclei of the individual members of the star results.

Other investigators, however, have interpreted the phenomenon differently. Schätzel (1932) states that the aggregates result from an entangling of flagella of the individual members. Riker *et al.* (1946) suggest that further work is necessary to determine the possibility of the bacteria being attracted by a particle with an electric charge different from that of the organisms. Other workers believe that the bacterial stars represent an attempt by these organisms to form colonies such as are found in certain of the algae and protozoa.

We have investigated, with the aid of an electron microscope, the various stages in the life history of *Phytomonas tumefaciens* when that organism developed in a special carrot medium. The chromatinic behavior within the star-shaped bodies was also studied by means of the Feulgen procedure and light microscopy. It is the purpose of the present paper to report our findings.

EXPERIMENTAL METHODS

The highly motile Chry. IIB strain of *Phytomonas tumefaciens* was used. It was grown on nutrient glucose agar for 18 to 20 hours and then inoculated heavily into the carrot broth of Stapp and Bortels (1931). The culture was permitted to incubate at room temperature for the desired period.

The carrot medium was prepared by first washing carrots in running tap water until all soil particles were removed. About 250 g of washed carrots were macerated in a meat chopper, placed in 500 ml of tap water, and cooked for 30 minutes in flowing steam. The carrot extract was filtered, and 200 ml of the juice were added to 1,200 ml of water. The solution was made slightly alkaline (pH 7.4) by the addition of sodium carbonate. Following the adjustment of the reaction, 0.2 g of $FeSO_4 \cdot 7H_2O$ and 0.2 g of $MnSO_4 \cdot 7H_2O$ in 100 ml of distilled water were added. The medium was placed in sterile test tubes to a depth of about three inches and sterilized intermittently in flowing steam on three successive days. During sterilization a heavy brown precipitate was formed. This was allowed to settle to the bottom of the tubes, and the clear supernatant was pipetted into sterile test tubes to a depth of about two inches. The medium was then ready for use.

In studying the chromatinic structure of the bacteria in the starlike cluster the following procedure was used. Films of the bacterial culture which had been grown on the carrot medium were made on clean glass slides. The preparations were fixed in osmic tetroxide vapor in a closed container for 20 minutes and then permitted to air-dry. The film was hydrolyzed in N/1 HCl at 58 C for periods varying from 5 to 40 minutes. Following hydrolysis the preparations were stained either by the usual Feulgen method or with Loeffler's alkaline methylene blue (Lewis, 1942; Dubos, 1945). The two procedures gave comparable results although differentiation of the chromatinic bodies and the cytoplasm was found to be somewhat sharper with the methylene blue stain. No counter stain was used. These preparations were examined with a light microscope.

Electron microscope preparations were made by placing a droplet of a diluted suspension of bacteria on the surface of the collodion-covered metal screens customarily used for electron microscopy. After standing for several minutes most of the droplet was withdrawn with folded lens paper. Care was taken not to touch the membrane with the paper. An RCA type EMC-1 instrument was used in the study.

EXPERIMENTAL RESULTS

Phytomonas tumefaciens, the causal agent of the crown-gall disease of plants, is described by Bergey *et al.* (1939) as a gram-negative bacterium that is 0.7 to 0.8 by 2.5 to 3.0 microns in size. The flagella are polar, as shown in figure 1, no. 1, and most strains are highly motile. When this organism is grown on nutrient glucose agar for 18 to 20 hours and then heavily inoculated into the iron manganese carrot medium, the bacteria are actively motile for the first few hours. During this period none of the characteristic star-shaped aggregates are found. At the beginning of the third hour, however, a delicate, slimy veil can be observed on or close to the surface of the medium. It is at this time that the aggregates begin to form.

If the process of star formation is observed by means of a specially constructed chamber, such as that described by Stapp and Bortels (1931), one finds that in the third hour individual bacterial cells come to rest at or close to the surface of the medium. Following this, in each case, a second organism moves into the vicinity of the quiescent bacterium and may either settle down next to the first or swim about it for a short period before coming to rest. A third organism, then a fourth, etc., follow the same procedure until the star-shaped bodies are formed. The aggregates may consist of as few as 2 or 3 organisms or as many as several hundred. It is believed probable, because of the behavior of the bacteria during the period in which the stars are initiated, that the force that brings these organisms together is chemotactic in nature.

Figure 1, no. 2, shows a very young bacterial star that is composed of 5 members. The preparation was obtained from a 4½-hour carrot broth culture and is incorporated here to show the relative position of the flagella. These structures were found to be at that end of the bacterium farthest from the center of the star. Since this species of bacteria has a single tuft of 3 to 6 flagella attached at only one end, this finding proves beyond reasonable doubt that the entangling of flagella of the individuals that comprise the aggregate is not a factor in star formation.

The bacteria within the aggregate characteristically elongate and within 24 hours may be two or three times their original length. Figure 1, no. 3, shows a 9-hour star in which elongation has already begun. In addition, this picture and no. 2 are interesting because they demonstrate the extrusion of a spherical mass of protoplasm from the bacterial cell. Thus far we have observed this phenomenon only at the ends of the bacteria farthest from the center of the aggregate. This may be due in part at least to the extreme difficulty of finding the star-shaped bodies at the proper stage of development and at the same time in such a position as to make the observation possible. Further consideration will be given to this phenomenon below. The organisms continue to elongate for several days, and we have on occasion observed individuals as long as 30 microns. This represents a tenfold increase in length. Most bacteria, however, show a maximal elongation of somewhat less than half that figure.

In addition to the increase in size, the organisms frequently show a pronounced swelling at the ends of the bacteria toward the center of the star. The swelling, which is well illustrated in figure 1, no. 4, may be observed as early as the twelfth hour, but is most pronounced after the second day.

The star-shaped aggregates become embedded in a slimy matrix very early in their formation and thereafter remain quiescent for a period ranging from 5 days to 3 weeks. Many hundreds of these bodies can frequently be found packed closely together in the gelatinous mass. The day following the formation of a star the individual members are bound together tenaciously. On repeated occasions these aggregates have been isolated, washed thoroughly in sterile media, and probed with microrods attached to a micromanipulator in an attempt to pull them apart. Despite intensive probing these bodies maintained their characteristic shape and only after prolonged effort was it possible to bring about their disintegration.

A typical 4-day-old bacterial star is shown in figure 2, no. 6. In this instance the five organisms radiate from a common center and adhere to one another only at their extreme tips. There is no evidence here of a fusion of the cells nor, as Stapp and Bortels (1931) have suggested, of a strand of protoplasm that con-

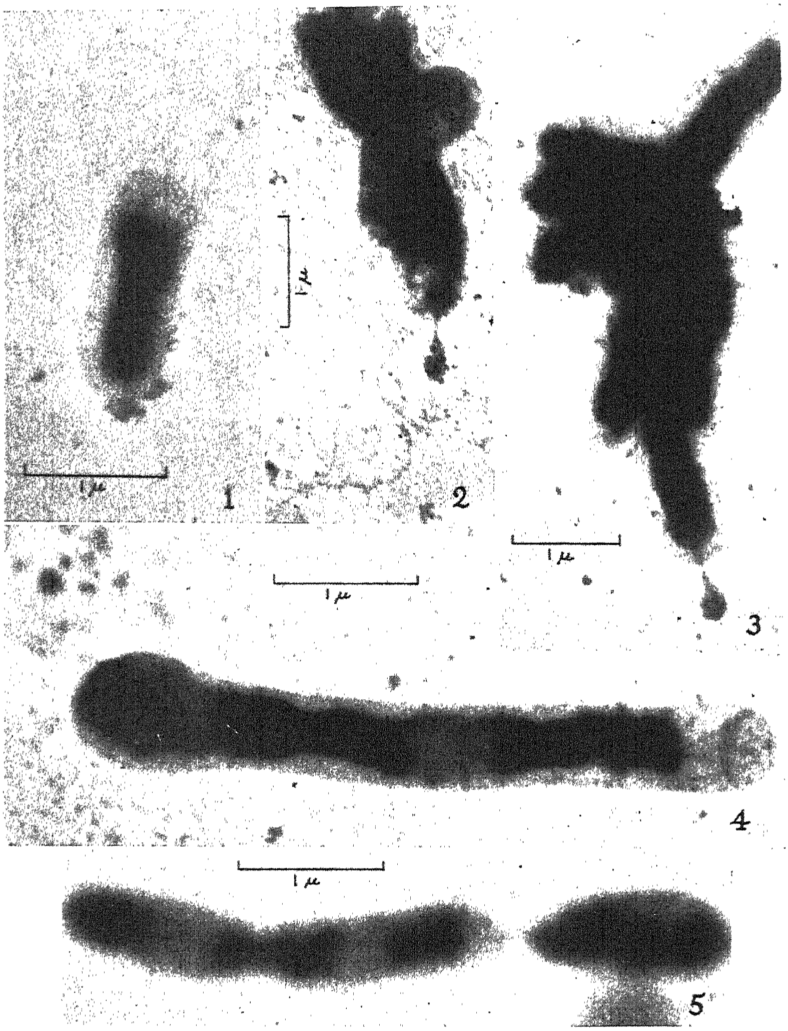

FIG. 1. No. 1. Electron micrograph showing arrangement of flagella in a typical young cell of *Phytomonas tumefaciens*. × 20,000 (Photograph by J. A. Carlile.)

No. 2. Electron micrograph of a 4½-hour bacterial star. The flagella are attached to that end of the organism farthest from the center of the aggregate. × 15,000 (Photograph by J. A. Carlile.)

No. 3. Electron micrograph of a 9-hour bacterial star showing an early stage of cellular elongation. The extrusion of a spherical mass of protoplasm from one of the cells is shown. The latter is also found in No. 2. × 15,000 (Photograph by J. A. Carlile.)

No. 4. Electron micrograph of a greatly elongated cell which has a characteristic swelling at one end. This type of cell is found following the dissolution of a bacterial star similar to that shown in No. 6. × 20,000 (Photograph by J. A. Carlile.)

No. 5. Electron micrograph of an elongated form in the process of division. The resulting organisms are similar to that shown in No. 1. × 20,000 (Photograph by J. A. Carlile.)

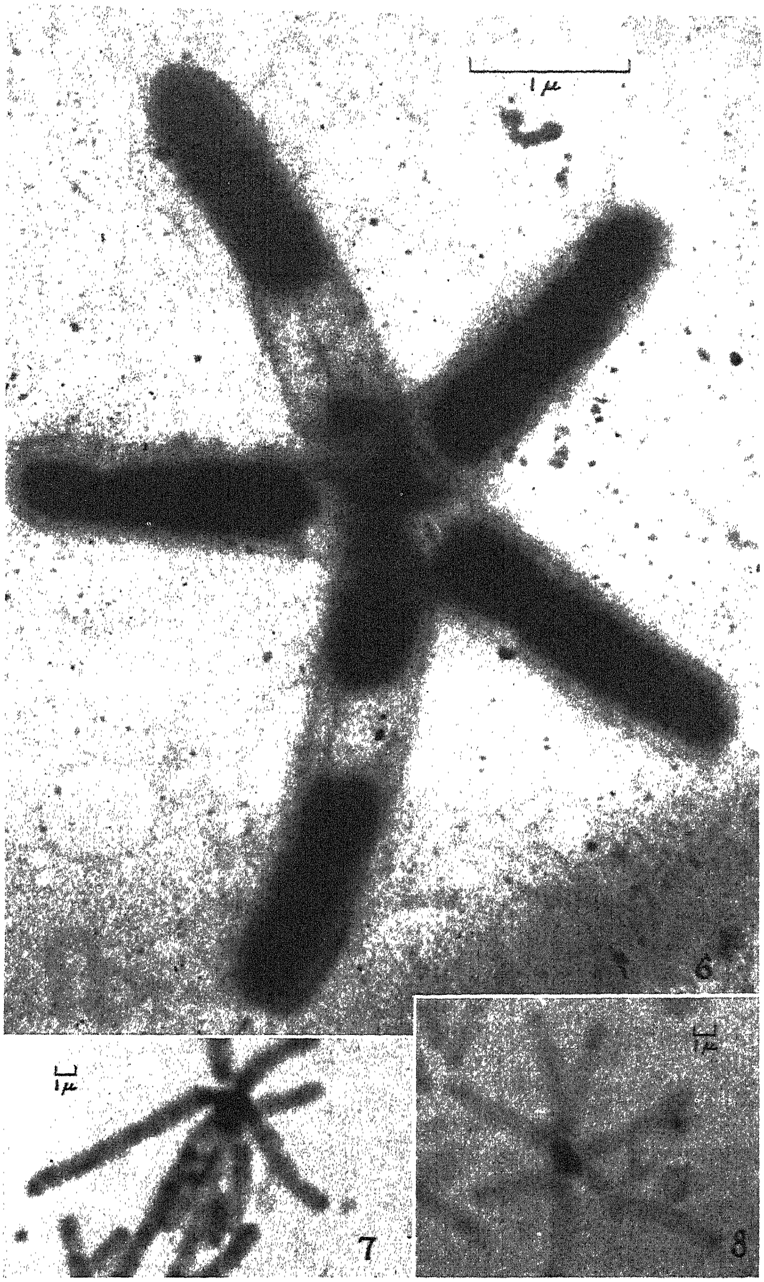

FIG. 2. No. 6. Electron micrograph of a 4-day bacterial star. Only a moderate elongation of the cells has resulted in this specimen. Note that the central mass has approximately the same opacity as the bacterial protoplasm. × 22,500 (Photograph by J. A. Carlile.)

No. 7. Light micrograph showing chromatinic material. The preparation was hydrolyzed in N/1 HCl at 58 C for 30 minutes. Loeffler's alkaline methylene blue stain was used. A concentration of chromatinic material is found at the center of the aggregate. × 3,950 (Photograph by J. A. Carlile.)

No. 8. Light micrograph showing a single, well-defined, Feulgen-positive body at the center of the bacterial star. The preparation was hydrolyzed at 58 C for 35 minutes and stained according to the Feulgen procedure. × 3,950 (Photograph by J. A. Carlile).

nects the individual organisms. The central portion of the aggregate appears to be occupied by a substance that shows approximately the same opacity as does the protoplasm of the bacteria themselves. Although the nature of this substance could not be determined with the electron microscope, we nevertheless feel confident that it is not foreign in origin but represents either a product of the bacterial cell, such as slimy mucilaginous material, or is perhaps bacterial protoplasm. This phase of the problem will be taken up in more detail below.

Toward the end of the quiescent period, which, as has been pointed out previously, is quite variable, a dissolution of the star-shaped bodies begins. Following disintegration the elongated components similar to that shown in figure 1, no. 4, float about in the medium and ultimately divide (no. 5) giving rise again to small motile forms (no. 1). Between the third and fourth weeks the culture no longer contains large numbers of starlike bodies but consists chiefly of small motile organisms, together with a lesser number of elongated cells. At this stage the individual organisms have the general appearance of those in the culture initially used. Thus, the cycle is completed.

The question of the significance of star formation in the life history of *Phytomonas tumefaciens* has recently been studied by Stapp (1942). This investigator has followed the behavior of the chromatinic substance within the aggregate, and he suggests that fusion of this material results. We have undertaken to reinvestigate this phase of the problem tinctorially using both the Feulgen procedure and Loeffler's alkaline methylene blue. Both methods were applied to hydrolyzed as well as unhydrolyzed specimens.

It was found that in many instances the Feulgen-positive material migrates to the end of the bacteria closest to the center of the star as shown in figure 2, no. 7. In other instances distinct nucleoids appear at both ends of the cells. This distribution of chromatinic material is evident not only in bacteria that comprise a star but also in certain elongated cells that are not members of an aggregate. In only a relatively few instances, however, have we found evidence of what appeared to be an actual fusion of the Feulgen-positive material of the various bacteria comprising a star. Figure 2, no. 8, shows one such example in which a single Feulgen-positive body is located directly in the center of a seven-membered star.

In the majority of instances, however, the central area appeared to be occupied by a substance that stained a very faint blue with the methylene blue stain. The staining reaction of this material was the same whether the preparations were hydrolyzed or stained without previous hydrolysis. The substance was probably bacterial slime. At times, however, one to several small granules that stained dark blue were found embedded in the lighter-staining matrix.

DISCUSSION

Attempts to explain the nature and possible significance of the so-called "star" phase in the life history of *Phytomonas tumefaciens* have given rise to a number of diverse concepts. These have been briefly outlined in the introduction.

We have reinvestigated the various possibilities suggested above by means of the electron microscope and the Feulgen staining technique. The electron microscope was found to be admirably suited for a study of the relative position of the flagella as well as for detecting the possible presence of a centrally located granule that might be instrumental in attracting the bacteria. The results indicate that the flagella are attached to that end of the bacteria farthest from the center of the star-shaped body and do not, therefore, appear to be directly involved in the formation of the aggregate. Furthermore, there was no evidence to indicate the presence of a centrally located granule of foreign origin that might serve to attract the bacteria and thus account for the formation of these bodies.

The comparison of the bacterial stars to certain colony types found in the algae and protozoa offers an interesting subject for speculation. Striking similarities as well as differences between these two kinds of bodies do, of course, exist and we believe that this interpretation cannot be ruled out until some other more plausible explanation for the origin and development of the bacterial stars is advanced.

The behavior of the chromatinic substance within the bacterial star is interesting and, as Stapp has indicated, there is a suggestion of a fusion of the Feulgen-positive material at the center of the aggregate.

We have observed many bacterial stars in which a concentration of the chromatinic substance was found in that part of the bacterial cell closest to the center of the star. The individual nucleoids were distinct, and they remained confined to the bacterial cell. We have also found some examples of what appeared to be actual fusion of the chromatinic material of the various bacteria comprising the aggregate. In these instances a single, large, well-defined, Feulgen-positive body was found directly in the center of the aggregate. These findings are, of course, very indicative of a simple form of sexuality in these bacteria.

No evidence has been found to indicate that a fusion of the bacteria comprising a star takes place, or that protoplasmic strands, such as those described by Stapp and Bortels (1931), bind the organisms together. The question that arises, therefore, is how the Feulgen-positive material which sometimes concentrates at the center of the aggregate leaves the bacterial cells. Although this question has not been definitely answered in the present study, we have found evidence to indicate that an extrusion of protoplasm from certain of the bacteria comprising newly formed stars takes place. This protoplasmic extrusion might be interpreted as being the result of plasmoptysis. However, plasmoptysis is usually associated with the sudden transfer of bacteria from a medium of high to one of very low osmotic value. It should be recalled that the carrot juice employed in this study was diluted with 6½ volumes of water, and therefore probably did not have an osmotic value greatly in excess of that found in the sterile tap water used for preparing the bacterial suspensions for the electron microscope studies.

Should the extrusion of protoplasm be found to occur at the ends of the bacteria closest to the center of the aggregate as well as at the opposite ends where we have observed it, then the presence of the centrally located Feulgen-positive material could be explained. If this were true it would also suggest a mechanism whereby a fusion of the protoplasm of the various bacteria comprising a star could result.

Because of the relatively few instances, however, in which we have observed what appears to be an actual fusion of the chromatinic material, we are reluctant to interpret the phenomenon as a conjugation at this time, and we believe that cytological studies alone will not suffice to clarify this question. It will be necessary to bring together in a single star different strains of the same species or individuals of closely related species and determine from this cross whether a recombination of characters results.

SUMMARY

The stages in the life history of *Phytomonas tumefaciens* as they occurred in the special carrot media of Stapp and Bortels have been studied by means of the electron microscope.

The diverse concepts that have been advanced in an attempt to explain the origin and nature of the star-shaped aggregates that are found in the developmental cycle of this organism have also been investigated. It is felt that certain of the interpretations given this phenomenon, i.e., the entangling of flagella of the various members of the aggregate and the attraction of the organisms by a foreign particle of opposite electric charge, can be abandoned on the basis of the work reported here.

A study of the chromatinic behavior within the bacterial stars revealed that in a small percentage of instances there was found what appeared to be a fusion of the Feulgen-positive material at the center of the aggregates. Although this finding is highly suggestive of a simple form of sexuality in these bacteria, it is nevertheless felt that cytological studies alone are inadequate to determine the answer to this question with certainty.

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THE ACCLIMATIZATION OF YEAST TO HIGH CONCENTRATIONS OF GLUCOSE: THE SUBSEQUENT EFFECT UPON ALCOHOL TOLERANCE

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In an earlier report by the writer (Gray, 1941) it was demonstrated that various strains and species of yeast vary widely in their abilities to tolerate alcohol in high concentrations. Four strains of *Saccharomyces cerevisiae* Hansen, the alcohol tolerances of which had been previously determined, were later shown (Gray, 1945) to vary also in their capacities to tolerate glucose in high concentrations, and it was pointed out that for these four strains, glucose tolerance varied directly with alcohol tolerance. In view of the possibility of a yeast strain possessing certain desirable characteristics (e.g., fast fermentation rate, etc.) but exhibiting low alcohol and glucose tolerances, it was felt desirable to determine if these tolerances could be raised by a process of acclimatization.

EXPERIMENTAL PROCEDURE

The yeast strain used throughout this work was *S. cerevisiae* Hansen (strain no. 1, Seagram yeast stock culture collection), and in all experiments the medium used was composed of 0.5 per cent KH_2PO_4, 0.7 per cent of Difco yeast extract, and glucose, the medium being adjusted with 0.1 N H_2SO_4 to a pH of 4.4 to 4.5. In previous work the medium employed consisted of 10 per cent yeast water plus glucose, but in the present work a medium with no suspended material was desired, so the first experiments were concerned primarily with the preparation of a clear medium from which glucose would be utilized as readily as from yeast water.

In the first experiment a simple medium consisting of 0.3 per cent Difco yeast extract (hereinafter referred to as DYE) plus glucose in concentrations ranging from 5.26 to 20.64 per cent was prepared. Twenty-five-ml lots of medium were inoculated with 0.5 ml of yeast suspension (0.25 ml freshly centrifuged yeast suspended in 1.0 ml of medium) and incubated without agitation at 30 C for 72 hours. At the end of the incubation period, glucose determinations were made by the method of Stiles, Peterson, and Fred (1926). Final glucose analyses showed that the percentage of glucose utilization was low, regardless of the initial glucose concentration. Because of the low glucose utilization this medium was obviously unsatisfactory, so attempts were made to improve it. With the thought that the medium might be deficient in phosphate, a series of fermentations were set up using media consisting of 0.3 per cent DYE and 22 per cent glucose, plus KH_2PO_4 in amounts varying from 0.01 to 1.0 gm per 100 ml. The results of 72-hour fermentations employing these media are presented in table 1 and figure 1.

The results in table 1 and figure 1 show that the percentage of glucose utilization from 0.3 per cent DYE medium can be increased by the addition of KH_2PO_4 in amounts ranging from 0.01 to 0.5 gm per 100 ml; the addition of greater amounts effected no further increase in glucose utilization.

A series of fermentations was then run with 0.3 per cent DYE and 0.5 per cent KH_2PO_4 medium plus varying amounts of glucose, in order to determine if the glucose tolerance was the same in this medium as it was in 10 per cent yeast water glucose medium. The results of this experiment are graphically illustrated in figure 2 and show that the latter medium is superior insofar as glucose utilization is concerned.

TABLE 1

The effect of KH_2PO_4 addition upon glucose utilization from 0.3 per cent DYE medium

(Initial glucose: 22.0 gm per 100 ml)

KH_2PO_4	FINAL GLUCOSE	GLUCOSE UTILIZATION
gm/100 ml	*gm/100 ml*	%
0.01	16.000	27.27
0.05	14.640	33.45
0.10	13.440	38.91
0.50	11.408	48.15
1.00	11.424	48.07

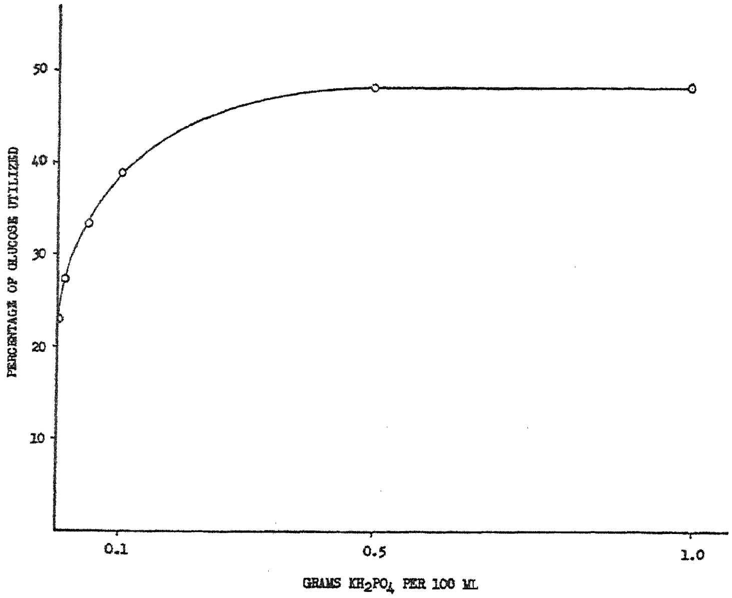

FIG. 1. THE EFFECT OF KH_2PO_4 CONCENTRATION UPON GLUCOSE UTILIZATION FROM 0.3 PER CENT DIFCO YEAST EXTRACT MEDIUM

Initial glucose: 22.0 gm per 100 ml.

Another series of fermentations was run to determine if increasing the DYE content would bring about an increase in the percentage of sugar utilization. In this experiment the medium consisted of 0.5 per cent KH_2PO_4, 10.128 per cent glucose, and DYE in amounts varying from 0.3 to 1.5 gm per 100 ml. The results obtained from 72-hour fermentations are presented in table 2.

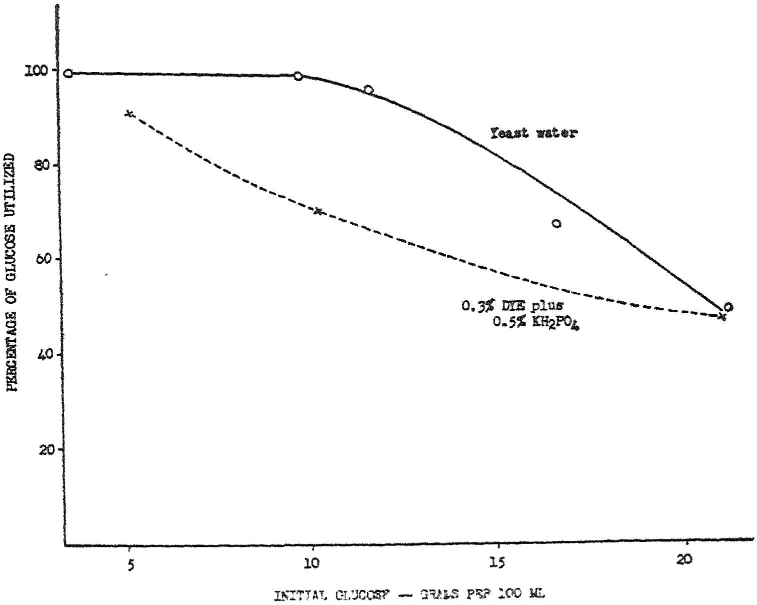

FIG. 2. GLUCOSE UTILIZATION FROM YEAST WATER MEDIUM AND MEDIUM CONTAINING 0.3 PER CENT DIFCO YEAST EXTRACT (DYE) PLUS 0.5 PER CENT KH_2PO_4

TABLE 2

The effect of DYE concentration upon glucose utilization

(Initial glucose: 10.128 per cent)

FERMENTER NO.	PER CENT DYE	INITIAL pH	FINAL GLUCOSE	GLUCOSE UTILIZED	AVERAGE GLUCOSE UTILIZED
			gm/100 ml	%	%
1	0.3	4.50	1.972	80.53	79.89
2	0.3	4.50	2.404	76.26	
3	0.5	4.43	0.328	96.36	94.84
4	0.5	4.43	0.676	93.32	
5	0.7	4.47	0.100	99.01	98.98
6	0.7	4.47	0.106	98.95	
7	1.0	4.50	0.118	98.73	98.79
8	1.0	4.50	0.117	98.84	
9	1.5	4.50	0.161	98.41	98.43
10	1.5	4.50	0.157	98.45	

From the results given in table 2 it may be seen that, by increasing the concentration of DYE, glucose utilization can be increased. Since the greatest percentage of utilization was from medium containing 0.7 per cent DYE, this concentration was employed for all subsequent work. Glucose tolerance was then determined for yeast no. 1 using a medium consisting of 0.5 per cent KH_2PO_4, 0.7 per cent DYE, and glucose in varying amounts, and it was found that the glucose tolerance was approximately the same as that obtained when 10 per cent yeast water glucose medium was used. Hence, in the following experiments all media were prepared using 0.7 per cent DYE and 0.5 per cent KH_2PO_4.

Acclimatization of yeast to alcohol. An attempt was made to acclimatize yeast to higher concentrations of ethyl alcohol than it can normally withstand by daily passage into fresh medium containing 12.5 per cent ethyl alcohol by volume and 11.3 gm of glucose per 100 ml. Transfers were made at 24-hour intervals, and at the end of 15 such transfers the yeast cells were no longer viable. It is possible that the yeast could have been acclimatized by daily passage into a gradually increasing alcohol concentration; however, in the present work no such attempt was made.

TABLE 3

A comparison of the glucose tolerances of acclimatized and nonacclimatized yeasts

(Based on 72-hour fermentations)

INITIAL GLUCOSE	PERCENTAGE OF GLUCOSE UTILIZED	
	Nonaccl. yeast	Acclimatized yeast
gm/100 ml		
5.28	98.79	98.72
9.94	98.54	97.66
15.23	84.39	89.96
20.40	69.70	75.84

Acclimatization of yeast to glucose. Acclimatization to high concentrations of glucose was accomplished by daily transfers to fresh medium containing 20 per cent glucose. After 27 such transfers the glucose tolerances of acclimatized and nonacclimatized yeast were determined. These determinations were made as previously described by inoculating 25-ml portions with 0.5-ml portions of yeast suspension (0.25 ml of freshly centrifuged wet yeast from a 24-hour-old culture, suspended in 1.0 ml of medium) and incubating for 72 hours. The results of these determinations, which are presented in table 3, show that the glucose tolerance of the acclimatized yeast was slightly higher than that of the nonacclimatized yeast.

The acclimatized culture was tested after 51 and 61 daily passages, and in both instances the results obtained were similar to those obtained with the culture which had received 27 transfers. In view of the possibility of these differences being due to differences in cell counts of inocula, several experiments were run in which the counts of the inocula were adjusted so that the suspension of acclimatized yeast had, in each experiment, approximately the same number of cells per ml as the suspension of nonacclimatized yeast. The results of these experiments, a summation of which is given in table 4, confirm the findings of the earlier experiments.

The effect of acclimatization upon alcohol tolerances. Since in earlier work it was shown that yeasts of normally high alcohol tolerance also exhibited high glucose tolerance as compared with yeasts of low alcohol tolerance, it might be reasonably assumed that since the glucose tolerance of yeast no. 1 had been raised, the alcohol tolerance would also be raised. A preliminary experiment was run to determine this point simply by setting up duplicate fermentations with acclimatized (61st passage) and nonacclimatized yeast in medium containing 9.13 per cent ethyl alcohol by volume. After 24 hours, glucose determinations were made and the percentage of glucose utilization was calculated. The results of this experiment are presented in table 5.

TABLE 4

The glucose tolerances of acclimatized and nonacclimatized yeasts

YEAST TYPE	NO. OF TRANSFERS	INOCULUM CELL COUNT	NO. OF REPLICATE FERMENTATIONS	INITIAL GLUCOSE	AVERAGE GLUCOSE UTILIZED
		millions/ml		*gm/100 ml*	%
Acclimatized	27	43	2	17.461	68.93
Nonacclimatized		43	2	17.461	65.75
Acclimatized	37	30	2	20.016	62.07
Nonacclimatized		30	2	20.016	58.38
Acclimatized	43	30	8	20.323	62.45
Nonacclimatized		30	8	20.323	58.73

TABLE 5

Alcohol tolerances of acclimatized and nonacclimatized cultures of yeast no. 1

FERMENTER NO.	YEAST	INITIAL GLUCOSE	INITIAL EtOH	24-HR GLUCOSE	GLUCOSE UTILIZED	AVERAGE GLUCOSE UTILIZED
		g/100 ml	*% by vol.*	*g/100 ml*	%	%
1A	Nonaccl.	1.440	9.13	0.348	75.83	72.99
1B	Nonaccl.	1.440	9.13	0.340	70.14	
2A	Accl.	1.440	9.13	0.674	53.19	53.26
2B	Accl.	1.440	9.13	0.672	53.33	
3A	Nonaccl.	1.440	0.0	0.033	97.74	
3B	Accl.	1.556	0.0	0.033	97.74	

This preliminary experiment showed that under conditions of an initial ethyl alcohol concentration of 9.13 per cent the nonacclimatized yeast was able to utilize 72.99 per cent of the glucose supplied during the 24-hour incubation period, whereas the acclimatized yeast utilized only 53.26 per cent. Contrary to expectations the culture of higher glucose tolerance did not exhibit a higher alcohol tolerance, but instead showed a lower alcohol tolerance. Since the inocula for this experiment were prepared in each case by adding 0.5 ml of yeast suspension (0.25 ml of wet yeast per ml of suspension), it was felt that another experiment should be run using inocula standardized on the basis of equal cell counts. In this experiment three different alcohol concentrations (8, 10, and 12 per cent) were employed and each 25-ml fermentation received 0.5 ml of yeast suspension which had a cell count of 185 million cells per ml. The initial glucose concentration was 1.446 gm per 100 ml; all media were adjusted to pH 4.5, and the fermentations were incubated for 24 hours at 30 C, after which time final glucose determinations were made. The results of this second experiment corroborated the findings of the first experiment in that the nonacclimatized yeast exhibited a higher alcohol tolerance, although its glucose tolerance was lower than that of the acclimatized yeast. Complete data from the second experiment are presented in table 6.

TABLE 6

Alcohol tolerances of acclimatized and nonacclimatized cultures of yeast no. 1

(Initial glucose: 1.446 gm per 100 ml)

FERMENTER NO.	YEAST TYPE	INITIAL EtOH	24-HR GLUCOSE	GLUCOSE UTILIZED
		% by volume	*gm/100 ml*	%
1	Nonaccl.	8	0.040	97.23
1A	Accl.	8	0.038	97.37
2	Nonaccl.	10	0.718	50.37
2A	Accl.	10	0.835	42.24
3	Nonaccl.	12	1.170	19.08
3A	Accl.	12	1.252	13.41
4	Nonaccl.	0	0.031	97.85
4A	Accl.	0	0.032	97.82

DISCUSSION

In the light of the foregoing results it seems obvious that the normal glucose tolerance of a yeast can be raised by the simple process of daily transfer to fresh medium of high glucose content. A similar method for acclimatizing a yeast to higher concentrations of alcohol than it can normally withstand was tried but found unsuccessful, although the possibility still exists that this might be accomplished by gradually increasing the alcohol concentration of the medium through a long series of cultures.

The most significant finding of the foregoing work was the fact that, although yeasts of normally high glucose tolerance also exhibit high alcohol tolerance, in the present instance when the glucose tolerance of yeast no. 1 was raised through a process of acclimatization, the alcohol tolerance was lowered. This problem is being further investigated with the view of making quantitative determinations of certain yeast cell constituents of both acclimatized and nonacclimatized yeast in order to determine what changes accompany the changes in alcohol and glucose tolerances during the acclimatization process.

SUMMARY

It has been found possible, by daily transfer to medium of high glucose concentration, to increase the glucose tolerance of yeast. Increasing glucose tolerance in this manner resulted in decreased alcohol tolerance.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NORTHERN CALIFORNIA-HAWAIIAN BRANCH

STANFORD UNIVERSITY MEDICAL SCHOOL, SAN FRANCISCO, CALIFORNIA, JUNE 22, 1946

A REPORT OF THE DETROIT NATIONAL MEETING OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS. *James Berry*, U. S. Western Regional Research Laboratory.

STREPTOMYCIN USE IN A CASE OF COLON BACILLUS MENINGITIS. *E. I. Sugarman*, Sugarman Laboratories, San Francisco.

A case report of colon bacillus meningitis which was treated successfully with streptomycin. This is the fourth case on record which has been treated successfully with this drug.

ENCEPHALITIS IN CALFORNIA. *E. H. Lennette*, Virus Laboratory, California State Department of Public Health.

A report on the encephalitis problem in California, and a presentation of the preliminary methods of study.

BIOLOGICAL AND BIOCHEMICAL FACTORS IN PENICILLIN PRODUCTION. *K. S. Pilcher*, Cutter Laboratories.

Of the biological factors influencing the yield of penicillin produced by fermentation, none is more important than the inherent capacity of the strain of mold employed to produce penicillin. Starting with strains isolated from natural sources, various investigators have been able to select mutant strains from spores irradiated with ultraviolet light or X-ray which are much superior to the parent strains. Strains now in use give yields of penicillin several-fold greater than those obtained with earlier strains.

In the fermentation medium commonly employed, corn steep liquor and lactose are the most important components. The corn steep liquor plays a complex role, providing both sources of energy and nitrogenous compounds needed in building cellular protoplasm. In addition, it supplies mineral elements, certain growth factors, and possibly a specific precursor of penicillin. The lactose provides a slowly fermented carbohydrate source which helps to stabilize the pH of the medium throughout the fermentation.

At least five naturally occurring penicillins have been identified. They have been called F, G, K, X, and di-hydro F. Other unidentified compounds probably exist.

SYNTHESIS OF DI- AND POLYSACCHARIDES WITH BACTERIAL PREPARATIONS. *M. Doudoroff*, Department of Bacteriology, University of California.

An elucidation and discussion of previously published material.

PROBLEMS ON GROWTH AND ASSIMILATION IN YEAST CULTURES. *C. E. Clifton*, Department of Bacteriology, Stanford University.

A discussion of some of the problems encountered and the factors involved in the growth and assimilation in yeast cultures.

EFFECT OF METHOD OF RECONSTITUTION ON THE PLATE COUNT OF POWDERED MILK. *E. R. Garrison*, Golden State Co., Ltd.

Bacterial plate counts were made on milk powders reconstituted in distilled water and in distilled water containing different concentrations of disodium phosphate ($Na_2HPO_4\cdot 12H_2O$), lithium hydroxide ($LiOH\cdot H_2O$), potassium fluoride ($KF\cdot 2H_2O$), and sodium citrate ($Na_3C_6H_5O_7\cdot 2H_2O$). The dilution bottles contained glass beads and were warmed to 45 C before using.

The milk powders were not always completely dispersed in distilled water and the powder particles interfered with the counting of bacterial colonies on the agar plates prepared from the lower dilutions. Disodium phosphate was unsatisfactory for reconstituting milk powder; a 1.25 per cent solution frequently failed to completely disperse the powder sample and the excessive foaming made pipetting difficult. Dilution water that contained 0.075 per cent lithium hydroxide or 0.75 per cent potassium fluoride usually gave good dispersion of the milk powders, but both solutions were definitely bactericidal. The best results were obtained by reconstituting the milk powder in a 1.25 per cent solution of sodium citrate since this solution usually completely dispersed the powder and showed no evidence of being bactericidal.

STUDIES ON THE "BACTERICIDAL" ACTION OF THE QUATERNARY AMMONIUM COMPOUNDS. *C. S. Mudge*, Department of Dairy Bacteriology, University of California at Davis.

A NEW BACTERIAL VITAMIN OCCURRING IN PLANT MATERIAL. *A. A. Andersen*, U. S. Western Regional Research Laboratory.

A new factor has been discovered while studying the nutritional requirements of certain undescribed bacteria isolated from spoiling tomato puree. The factor is not present in nutrient glucose agar. Sixteen known vitamins would not replace it. It is widely distributed in organic nature, having been detected in many fruits, vegetables, yeasts, molds, and bacteria. All plant materials tested were found to contain the active factor except in a few cases in which the presence of antibiotic material obscured its demonstration.

The factor is stable to autoclaving and to sunlight through glass. It is soluble in water, ethanol, and other organic solvents, in varying degrees. It is dialyzable, not precipitated by lead, silver, or barium salts, and is unaffected by hydrogen sulfide. It is adsorbed on charcoal, better at pH 7.0 than 4.0 or 10.0. Elution is not effected with dilute or concentrated ammonium hydroxide, but is effected with ethanol-water-ammonia (70:25:5). It is not adsorbed on fuller's earth from aqueous solution but is adsorbed from ethanol or other organic solvents by adsorbents of this type. Magnesium oxide, supersorb, florex, kaolin, permutit, and alumina adsorb the activity from solvents higher in the series than ethanol, which serves as an eluting agent. Its properties differentiate it from the vitamins and growth factors described in the literature.

It appears that cultural methods, using standard media, may be inadequate in the examination of foodstuffs, naturally containing the factor, and possibly harboring in consequence an extensive bacterial flora which apparently escapes detection in routine tests.

CULTIVATION OF THE MURINE SK STRAIN OF POLIOMYELITIS VIRUS IN DEVELOPING EGGS. *Edwin W. Schultz and John B. Enright*, Department of Bacteriology and Experimental Pathology, School of Medicine, Stanford University, California.

The mouse-adapted SK strain of poliomyelitis virus described by Jungeblut and Sanders (J. Exptl. Med., 1940, *72*, 407) has been carried through 14 passages in developing eggs without evident diminution in the concentration of virus. Multiplication of the virus was associated with a high incidence of deaths in the embryos between the second and fifth days. The incidence was higher in inoculated eggs incubated at 35 C (80 per cent) than in eggs incubated at 37 C (40 per cent). Virus from the tenth passage was neutralized by murine SK antiserum in significant dilutions.

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